Analysis of *In Vitro* Growth and Characterisation of Metabolic Pathways of *Mycoplasma suis* Linked to Pathogenesis

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1. Summary

The haemotrophic bacterium *Mycoplasma suis* is the causative agent of infectious anaemia in pigs (IAP), a worldwide and economically significant disease. The acute disease is characterised by severe anaemia, high bacteraemia, fever and high-grade hypoglycaemia. Antibiotic treatment using oxytetracyline protects the pigs against death, but does not eliminate the bacterium from the host. Hence, chronic low-grade *M. suis* infections are predominant. Clinical signs of the chronic form are mild anaemia, growth retardation in piglets, decreased reproductive efficiency in sows, and increased susceptibility to other infections.

The crucial barrier to systematic analyses of the biology of *M. suis* is that *M. suis* as well as all known haemotrophic mycoplasmas has not been cultivable *in vitro* so far. Thus, *M. suis* research still relies on the splenectomised pig model, and on animal-to-animal passages, a method connected with serious ethical concerns. In this Ph.D. thesis presented, I describe efforts to establish an *in vitro* cultivation system for *M. suis* as a model system for all haemotrophic mycoplasmas with the aim to replace animal experiments. I attempted *M. suis* propagation by inoculating different *Mycoplasma* media with anti-coagulated blood from experimentally infected and acutely diseased pigs, and by systematically diversifying proved culture techniques for mycoplasmas. Some cultivation approaches used led to a kind of maintenance of *M. suis* over 12 weeks in liquid *M. suis* cultures. Scanning electron microscopic analysis of subcultured *M. suis* cells on agar plates provided evidence: for the first time, propagated *M. suis* cells outside the natural host i.e. the pig. These propagated *M. suis* were packed in dense microcolonies consisting of small irregular *M. suis* nanocells indicating a kind of nanotransformation. Interestingly, these unique *M. suis* nanoforms are able to infect healthy porcine erythrocytes *in vitro,* thus indicating viability and infectivity.

One main clinical sign of acute IAP is severe and often life-threatening hypoglycaemia caused by glucose consumption of *M. suis* itself. Due to the not-yet-established *in vitro* culture system and to missing whole genome sequences, little was known about the metabolism and the transport capacities of *M. suis*. Therefore, the second part of the Ph.D. thesis presented aimed to explore this part of the *M. suis* biology in more detail. For this, a random shotgun screening approach in combination

with subsequent Southern blot hybridisation of genomic *M. suis* DNA was used. Thereby the transport system for the glucose uptake (i.e. the phosphoenolpyruvate:sugar phosphotransferase system PTS^{glc} uptake system), the pre-protein secretion machinery (Sec-translocase), the phosphate (P_i)-specific import system (Pst-transporter) and several glycolytic enzymes of *M. suis* were identified and their genomic context was characterised. The genomic organisation of most of the genes identified was analogous to other mycoplasmas. Only the organisation of the Pst-transporter differs from other mycoplasmas. In *M. suis*, PhoU, the putative negative regulator of the Pho regulon, is not encoded together with the subunits of the Pst-transporter, whereas in other mycoplasmas, the *pst*-genes are organised in a polycistronic *pst*SCAB-*pho*U operon.

Adhesion to erythrocytes is one of the main characteristics of haemotrophic mycoplasmas. Electron microscopic investigations revealed that the adhesion is mediated by fine bacterial fibrils. Recent studies have shown that a glycolytic enzyme i.e. the GAPDH protein MSG1 is involved in the adhesion. Within the present Ph.D. study a second glycolytic protein i.e. α-enolase of *M. suis* was identified by the above shotgun screening, and further characterised as potential adhesion and virulence factor. The *M. suis* α-enolase was expressed in *Escherichia coli* and the resulting purified recombinant protein was used to raise an anti-*M. suis* α-enolase rabbit serum and to perform characterisation assays. Interestingly, despite the missing classical signal sequence for export, *M. suis* α-enolase is a membrane-localised and surface-accessible protein with immunogenic and adhesive properties. Further studies are necessary to evaluate the role of *M. suis* α-enolase as a putative part of the *M. suis* adhesion complex.

2. Zusammenfassung

Mycoplasma suis gehört zur Gruppe der nicht-kultivierbaren, hämotrophen Mykoplasmen. *M. suis* verursacht beim Schwein die porcine infektiöse Anämie (IAP), eine weltweit verbreitete Erkrankung von erheblicher ökonomischer Bedeutung. Die akute Verlaufsform ist gekennzeichnet durch schwerwiegende Anämie, hochgradiger Bakteriämie, Fieber und lebensbedrohender Hypoglykämie. Mittels antibiotischer Behandlung mit Oxytetrazyklin können betroffene Schweine von klinischen Symptomen geheilt werden, jedoch ist eine vollständige Erregereliminierung nicht möglich. Infolgedessen findet man gehäuft chronische *M. suis* Infektionen. Symptome der chronischen Verlaufsform sind geringgradige Anämie, Wachstumsretardierung bei Ferkeln, sinkende Fortpflanzungsleistung bei Sauen sowie eine gesteigerte Anfälligkeit für andere Infektionen.

Eine systematische Aufklärung der *M. suis* Pathobiologie ist aufgrund der Unkultivierbarkeit des Erregers stark beeinträchtigt. Für die Vermehrung von *M. suis* wird nach wie vor das splenektomierte Schweinemodell verwendet, eine Methode mit ernsthaften ethischen Bedenken. Die vorliegende Ph.D. Arbeit beschäftigt sich mit der Etablierung eines *in vitro* Kultursystems für *M. suis,* das als Ersatz für die Vermehrung im Tiermodell und als Modellsystem für alle hämotrophen Mykoplasmen dienen soll. Die Vermehrung von *M. suis* wurde in verschiedenen Mykoplasmen-spezifischen Medien durchgeführt, die mit antikoaguliertem Blut von experimentell infizierten und akut erkrankten Schweinen inokuliert wurden. Ausserdem wurden die für Mykoplasmen geprüften Kulturtechniken systematisch verändert. In mehreren Kulturansätzen in Flüssigmedien konnte erfolgreich eine Art *in vitro* „Maintenance“ erreicht werden: über einen Zeitraum von 12 Wochen konnte *M. suis* auf einem konstanten Niveau in den Kulturen nachgewiesen werden. In Subkulturen aus diesen Kulturen auf festen Nährmedien konnten in rasterelektronenmikroskopischen Aufnahmen erstmals vermehrte *M. suis* Zellen ausserhalb des Wirts nachgewiesen werden. Es waren dicht gepackte Mikrokolonien aus kleinen, unregelmässigen *M. suis* Organismen sichtbar, welche erste Hinweise auf eine Art Nanotransformation von *M. suis* lieferten. Interessanterweise waren diese *M. suis* Nanoformen fähig, porcine Erythrozyten *in vitro* zu infizieren. Dies ist ein Indiz für die Viabilität und Infektiosität dieser *M. suis in vitro* Kulturformen.

Ein typisch klinisches Symptom der akuten IAP ist eine schwerwiegende, oft lebensbedrohliche Hypoglykämie, die durch den Glukoseverbrauch von *M. suis* selbst verursacht wird. Aufgrund des fehlenden Kultursystems und bis vor kurzem unbekannter Genomsequenzen war bislang wenig über den Metabolismus und die Transportsysteme von *M. suis* bekannt. Daher wurde im zweiten Teil der vorliegenden Ph.D. Arbeit dieser Bereich der *M. suis* Biologie näher beleuchtet. Dazu wurden *M. suis* Genombibliotheken sequenziert und nachfolgend Southern Blot Hybridisierungen von genomischer *M. suis* DNA durchgeführt. Dabei wurden das Transportsystem für die Glukose-Aufnahme (Phosphoenolpyruvate:Zucker Phosphotransferase PTS^{glc} System), die Präprotein-Sekretionsmachinerie (Sec-Translokase), ein Phosphat (P_i)-spezifisches Importsystem (Pst-Transporter) und verschiedene glykolytische Enzyme von *M. suis* identifiziert und ihr Genomkontext charakterisiert. Dabei zeigte sich, dass die Genomorganisation von *M. suis* bei den meisten der untersuchten Gene eine hohe Analogie zu der von anderen Mykoplasmen aufweist. Nur die Organisation des Pst-Transporters unterscheidet sich in *M. suis* von anderen Mykoplasmen. In *M. suis* ist phoU, der putative negative Regulator des Pho-Regulon, nicht in unmittelbarer Nähe zu den anderen Untereinheiten des Pst-Transporters lokalisiert, wohingegen in anderen Mykoplasmen die *pst*-Gene in einem polycistronischen *pst*SCAB-*pho*U-Operon organisiert sind.

Eine wichtige Eigenschaft hämotropher Mykoplasmen ist die Adhäsion an Erythrozyten. Elektronenmikroskopische Untersuchungen haben gezeigt, dass feine fibrilläre Strukturen die Anheftung von *M. suis* an die Erythrozytenmembran vermitteln. Studien haben gezeigt, dass ein glykolytisch aktives Enzym, das GAPDH von *M. suis* (MSG1) an der Adhäsion beteiligt ist. In der vorliegenden Ph. D. Arbeit wurde die α-Enolase, ein zweites Glykolyse-Enzym von *M. suis*, mittels Sequenzierung einer Gen-Bibliothek identifiziert und als potentieller Adhäsions- und Virulenzfaktor charakterisiert. Die *M. suis* α-Enolase wurde in *Escherichia coli* rekombinant hergestellt. Mit Hilfe des rekombinanten Proteins wurde ein anti-*M. suis* α-Enolase Kaninchen-Immunserum generiert und die Eigenschaften der *M. suis* α-Enolase näher charakterisiert. Dabei konnte nachgewiesen werden, dass die *M. suis* α-Enolase trotz fehlender Signalsequenz für Export und Insertion ein Membran-lokalisiertes, oberflächenständiges Protein mit immunogenen sowie adhäsiven

Eigenschaften ist. Weitere Untersuchungen sind notwendig, um die genaue Funktion der *M. suis* α-Enolase in einem komplexen *M. suis*-Adhäsionsapparat zu definieren.

3. Introduction

3.1 The genus Mycoplasma

Mycoplasmas are cell wall-less pleomorphic bacteria that live in strict association with eukaryotes either extracellular closely attached to host cells or intracellular. In general, they are highly adapted mucosal pathogens on the epithelia of the respiratory or urogenital tract where they survive as parasites in close association with their host cells. Mycoplasmas are among the smallest self-replicating organisms known so far and are characterised by strikingly small genomes. These genomes consist of a single circular genome ranging from ~580 kb for *Mycoplasma genitalium* to ~1360 kb for *Mycoplasma penetrans* [Sasaki et al., 2002; Fraser et al., 1995]. Mycoplasmas, in particular *M. genitalium,* have therefore attracted much attention as model organisms for the investigation of the biology of minimal cells [Suthers et al., 2009; Gibson et al., 2008; Glass et al., 2006]. According to the significant genome reduction their metabolic potential, i.e. all biochemical reactions which these organisms can perform autonomously, is extremely reduced and tailored towards the needs of a parasitic lifestyle closely associated with and adapted to their host cell and the corresponding host environment. Adapting to the resources available in its hosts they have eliminated “unnecessary” genes including those responsible for oxidative phosphorylation, ATP production via pentose phosphate pathway, and the biosynthesis of amino acids, nucleotides, lipids, and cofactors reflecting its high metabolic host adaptation [Baranowski et al., 2010; Kuhner et al., 2009; Suthers et al., 2009].The dominating hypothesis is that mycoplasmas evolved by a degenerate or reductive evolution from gram-positive bacteria [Messick, 2004]. They are characterised mainly by a low G+C content of the DNA (23 - 33 mol %), and by an alternative codon usage: The common stop codon UGA is translated into the amino acid tryptophan. Due to the reduced genome and numerous nutritional requirements for biosynthetic precursors such as lipids (i.e. sterol), amino acids and fatty acids make them dependent on the host cell. In spite of *Mycoplasma* media available only a minority of the mycoplasmas existing in nature have been cultivated so far [Razin et al., 1998].

3.1.1 Pathogenicity

Mycoplasmas usually exhibit a strict host and tissue specificity. Pathogenic human and animal mycoplasmas are primarily connected with diseases of the respiratory tract (*M. pneumoniae*, *M. penetrans, M. hyopneumoniae, M. pulmonis*), the urogenital tract (*M. genitalium*, *M. hominis, M. penetrans*) or the joints (*M. arthritidis, M. hyosynoviae*). The adhesion of mycoplasmas to host cells is the crucial step for successful colonisation and survival [Razin et al., 1998]. Several pathogenic mycoplasmas, e.g. *M. genitalium*, *M. pneumoniae* and *M. penetrans* form flask-shaped cells expressing attachment proteins as a specialised terminal tip-like structure to adhere to, and penetrate host target cells [Boguslavsky et al., 2000; Baseman, 1996; Jensen, 1994; Lo et al., 1993]. This tip organelle is a network of primary adhesin molecules (e.g. P1, P30 adhesins of *M. pneumoniae*) and several accessory membrane proteins that act in concert with cytoskeletal elements [Baseman, 1996]. Some of these *Mycoplasma* species (e.g. *M. mobile* and *M. pneumoniae*) are also capable of gliding on solid surfaces. The mechanism of this peculiar property is still unknown [Pich et al., 2006; Hasselbring et al., 2005; Razin et al., 1998].

Invasion and intracellular replication is one mycoplasmal way to efficiently persist and circumvent the host immune system. Other possible defence mechanisms evolved by mycoplasmas are molecular mimicry masking host proteins as antigens and phenotypic plasticity, i.e. antigenic variation. This reversible antigenic alteration of surface components enhances the colonisation of host tissues and allows for an effective avoidance of immune recognition [Rottem, 2003].

3.2 Mycoplasma suis*, a haemotrophic* Mycoplasma

3.2.1 Taxonomy

M. suis belongs to the group of haemotrophic bacteria which were formerly classified into the two haemotrophic genera *Haemobartonella* and *Eperythrozoon* within the order *Rickettsiales*. Based on the phylogenetic sequence analysis of the 16S rDNA and *rpo*B sequences as well as their phenotypic characteristics (e.g. lack of cell wall, small size, resistance to penicillin, and susceptibility to tetracyclines), the genera *Eperythrozoon* and *Haemobartonella* were reclassified into the family *Mycoplasmataceae* within the class *Mollicutes* (latin: *mollis*, soft; *cutis*, skin) [Tasker

et al., 2003; Messick et al., 2002; Neimark et al., 2002; 2001; Rikihisa, 1997]. Now the haemotrophic mycoplasmas (HM) represent a distinct new cluster within the genus *Mycoplasma* and have been given the trivial name of “haemoplasmas”. The currently known HM species were found in mouse and rat (*M. coccoides*; *M. haemomuris*), in dog (*M. haemocanis*), cat (*M. haemofelis*), sheep and goat (*M. ovis*), cattle (*M. wenyonii*), and in pig (*M. suis*). Over the past years there emerged a growing list of new candidates (e. g. in cat *Candidatus* M. turicensis and *Candidatus* M. haemominutum; in llama *Candidatus* M. haemolamae, in cattle *Candidatus* M. haemobovis) [Hoelzle et al., 2011; Hoelzle, 2008; 2007e; Messick, 2002].

3.2.2 Significance and transmission of *M.suis* infections

M. suis infections are distributed worldwide and of serious economic significance for the pig industry [Hoelzle et al, 2010; Ritzmann et al., 2009]. The resulting disease also referred to as infectious anaemia in pigs (IAP) is the most frequent porcine infectious anaemia. Comprehensive prevalence data are rare due to difficulties in diagnosis. A recent study from Germany examined the prevalence of *M. suis* in slaughter pigs (20 - 30 kg) by using a quantitative real-time Light Cycler-PCR (qLC-PCR) based on the *msg*1-gene of *M. suis* [Ritzmann et al., 2009; Hoelzle et al., 2007c]. Thereby, *M. suis* occurred in 13.9% (164/1176) of tested individuals as well as in 40.3% (79/196) of all pig farms. There was recently evidence that *M. suis* is not only prevalent in domestic pigs but also present in wild boars. Notably, individuals in wild boar enclosures are much more concerned by *M. suis* infections due to higher population density and closer contact to each other. The wild boar as *M. suis* carrier and reservoir can be considered [Hoelzle et al., 2010].

M. suis is not found in nature as free-living organisms. The main transmission route is zoo technical manipulations by farmers or veterinarians e.g. through contaminated needles and surgical equipment [Henderson, 1997]. Experimental transmission was achieved by various blood-sucking arthropod vectors including lice (*Haematopinus suis*), mosquitoes (*Aedes aegypti*), and stable flies (*Stomoxys calcitrans*) to pigs [Prullage et al., 1993; Heinritzi, 1992]. Natural transmission may occur vertically across the placenta, or horizontally via lesions due to social ranking fights. In general, close contact with infected individuals is thought to be the principal source of

transmission between animals, or from animals to humans [Hoelzle, 2007d; Yang et al., 2000]. Several case reports recently described HM-like infections in humans. However, the zoonotic relevance is yet unclear. Human *M. suis*-infections were detected in a large proportion of swine farm workers (49% of tested persons) in Shanghai, China after intimate contact with *M. suis*-diseased pigs [Yuan et al., 2009].

3.2.3 Microbial properties

M. suis is a cell wall-less pleomorphic bacterium which occurs rod-shaped, spherical, or ring-shaped. In stained blood smears (acridine orange-stained or Wright-Giemsa-stained) *M. suis* is visible individually or in chains across the surface of pig erythrocytes (Figure 1). The organisms are also found free in the plasma [Neimark et al., 2001; Zachary and Basgall, 1985] or intracellular in erythrocytes (Figure 2) [Groebel et al., 2009].

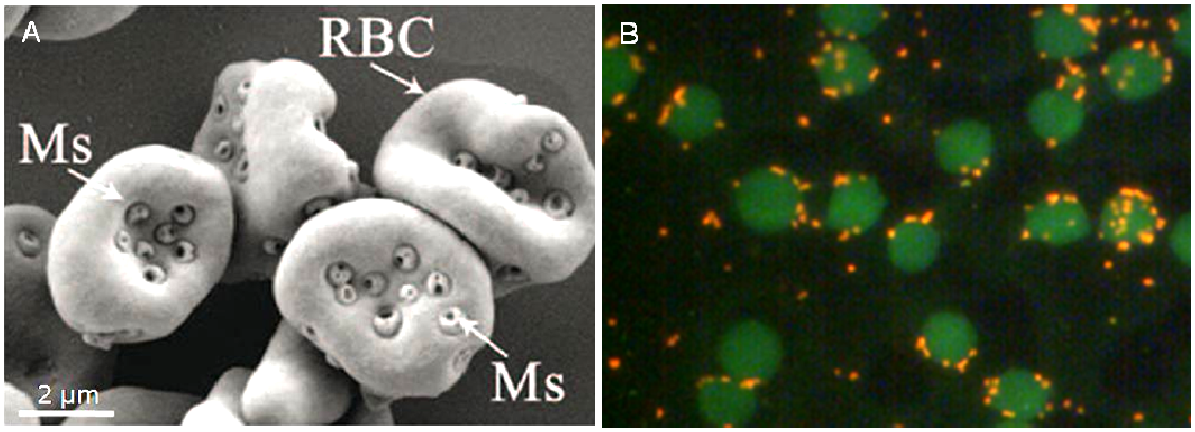

Figure 1. Intimate association between *M. suis* (Ms) cells and porcine red blood cells (RBCs). (A) Scanning electron micrograph (SEM); (B) Acridine orange-stained blood smear of an *M. suis*-positive piglet. Many *M. suis* cells are found attached to the surface of RBCs, marked in orange. Figure according to [Groebel et al., 2009].

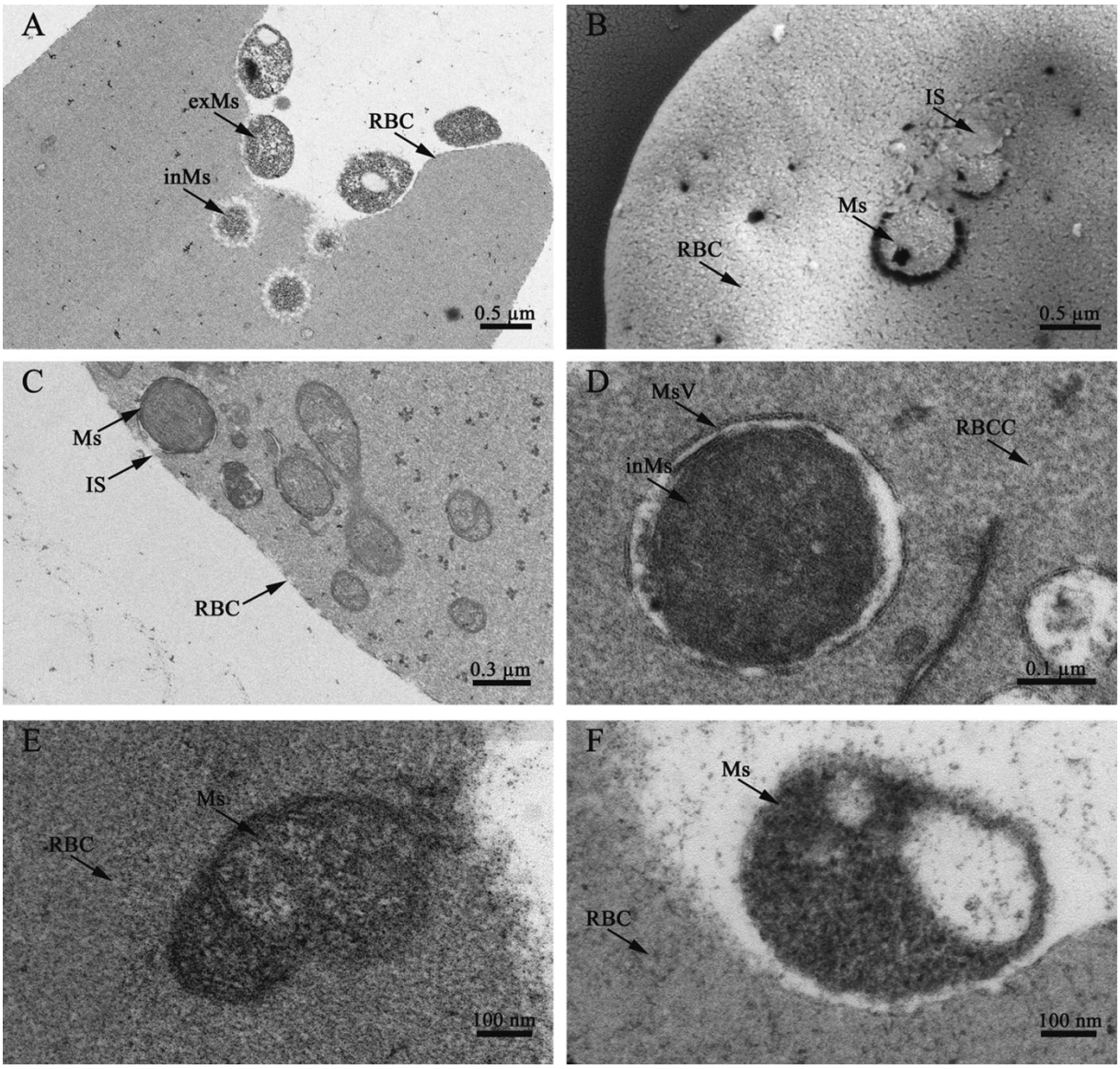

Figure 2. Invasion of porcine RBCs by *M. suis* 08/07 shown by SEM (B) and transmission electron microscopy (TEM; A and C to F). During invasion *M. suis* 08/07 is located in deep invaginations of the RBC membrane (A). As invasion progresses, the RBC membrane conforms to the shape of the bacterial cells (A and C), and newly formed membrane material covers the surface of the bacteria (B and C). As a result, invading mycoplasmas are located within an intraerythrocytic vacuole (D). Intracellular forms that are free in the RBC cytoplasm (E) have a shape and size similar to the shape and size of extracellular attached *M. suis* cells (F). exMs, extracellular *M. suis*; inMs, intracellular *M. suis*; IS, invasion scar; Ms, *M. suis*; MsV, *M. suis* vacuole; RBCC, RBC cytoplasm. Figure according to [Groebel et al., 2009].

M. suis parasitizes on the surface of the erythrocytes in an unique life-cycle and it is evident that these agents also replicate on RBCs. Electron microscopic investigations demonstrated at least four different replication forms (small coccoid immature form, juvenile discoid form, mature ring-shaped form) on the RBC surface indicating that *M. suis* multiplies on the RBC surface [Zachary and Basgall, 1985]. *M. suis* possesses a circular chromosome which is composed of 709 kb (*M. suis* strain KI_3806) to 742 kb (*M. suis* strain Illinois) with a 31% GC-content [Oehlerking et al., 2011; Messick et al., 2011]. These relatively small genomes and the low GC contents range within the published values for mycoplasmas. The *M. suis* genome differs from other *Mycoplasma* genomes by the high portion of predicted proteins without functional assignment (62%) which may reflect the adaptation of *M. suis* to a special environment. Furthermore, rather limited transport and metabolic capacities were found in the genome. Obviously, *M. suis* generates ATP through glycolysis. Tricarboxylic acid cycle, arginine hydrolysis, and urea hydrolysis are incomplete or missing.

3.2.4 Infectious anaemia in pigs

The *M. suis*-caused disease known as infectious anaemia in pigs (IAP) can affect pigs of any age. For acute affection-determining factors i.e. weaning, birth, periparturient period, sickness, viral infections, and other types of stress seem to be predisposing [Gresham et al., 1994; Heinritzi, 1990]. An acute disease outbreak is preferentially found in affected individuals after distinct stress situations such as birth, post weaning, after zoo technical manipulations or rank fights [Hoelzle, 2008; Smith et al., 1990]. Acute *M. suis* infections are characterised by high bacteraemia, immune-haemolytic icteroanaemia, increase of body temperature of up to 42°C, acrocyanosis and strong hypoglycaemia (Figure 3) [Hoelzle, 2008]. Due to the radical decrease in the blood glucose concentration, acutely infected pigs are at risk of going into a coma followed by death [Hoelzle, 2008; Smith et al., 1990]. Growth rate decreases in pigs that survive the acute phase of the disease. In sows, infection may lead to pyrexia, anorexia, depression, decreased milk production and poor maternal behaviour [Heinritzi, 1992; Zinn et al., 1983; Brownback, 1981; Henry, 1979].

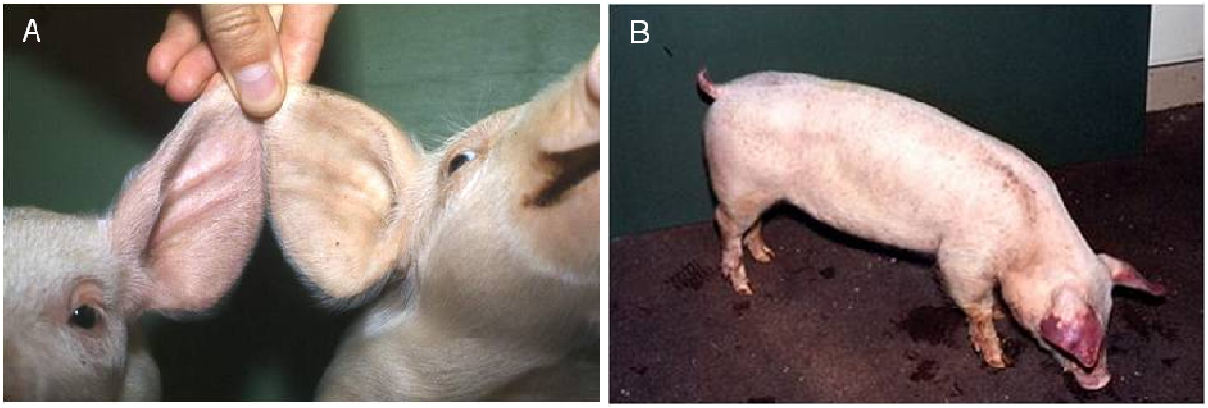

Figure 3. Clinical IAP symptoms are (A) immune-haemolytic icteroanaemia and (B) cyanosis, in most cases at the ear tips (pictures Clinic for Swines, Ludwig Maximilian University, Munich, Germany).

Today, the significance of the acute form of IAP has declined since antibiotic treatment of pigs e.g. with oxytetracycline could be used as a therapeutic measure. Nonetheless, pigs still may become persistently infected asymptomatic carriers [Messick, 2004; Smith, 1990; Heinritzi, 1990]. Chronic low-grade *M. suis* infections are of economical significance because they are often accompanied by other infectious diseases of the respiratory and/or the digestion tract. To date, it is unclear to what extent *M. suis* infections represent a trigger in the manifestation of other disease complexes or appear as a secondary infection. Clinically chronic *M. suis* infections occur either as asymptomatic infections or wide ranging complexes of symptoms i.e. unthriftiness, pallor, and sometimes allergic skin reactions, poor reproductive performance in sows (e.g. irregular cycles, anestrus, abortion, small litters, premature births, stillbirths and the birth of weak piglets) [Schweighardt et al., 1986; Zinn et al., 1983; Brownback, 1981; Henry, 1979].
Once pigs have been infected with *M. suis* they remain lifelong carrier animals even after clinical signs have resolved. Despite an intense immune response and even after antibiotic treatment it is impossible to eliminate *M. suis* from the host [Hoelzle, 2008; Messick, 2004]. These carriers are important regarding the eradication and epidemiology of *M. suis* infections.

3.2.5 Pathogenicity and virulence factors

Current knowledge about pathogenicity in *M. suis* infections is rather limited. This can primarily be led back to the lack of an *in vitro* culture system. Nevertheless, few

pathogenic features are described for *M. suis*. For instance, it is well defined that the adhesion to its host cell, to the erythrocyte, is a crucial step in the lifecycle of *M. suis*. Furthermore, new isolates were detected which were able to invade erythrocytes. This intracellular parasitism is obviously associated with virulence enhancement and signals high capability to adaptation of *M. suis* [Groebel et al., 2009]. The contact between host cell and pathogen is rather intimately as shown in several electron microscopic investigations [Groebel et al., 2009; Zachary and Basgall, 1985; Pospischil and Hoffmann, 1982]. This interaction is obviously attended by an irreversible deformation of the RBC surface and by structural alterations of the cytoskeleton in terms of compression and actin rearrangement. Within these deformed membrane areas *M. suis* organisms are intimately connected with, but distinctly separated from the membrane by a 30-nm electron lucent zone. Fine bacterial fibrils that are obviously responsible for the intimate attachment to the erythrocyte can be observed in scanning electron micrographs (Figure 2F). The drastic transformation of the erythrocyte membrane is held responsible for the decreasing osmotic resistance of erythrocytes, as well as for the transient induction of cold-reactive anti-erythrocyte IgM auto antibodies (cold agglutinins) as known from *M. pneumoniae*, *M. synoviae*, *M. gallisepticum* [Chian and Chang, 1999; Sahu and Olson, 1976; Kuniyasu and Yoshida, 1972; Feizi and Taylor-Robinson, 1967]. Concomitantly, peaking levels of warm-reactive IgG auto antibodies can be detected in the blood masking the *M. suis*-specific immune response [Felder et al., 2010; Hoelzle et al., 2006]. Porcine ß-actin was recently identified as one of the target antigens of these warm IgG auto antibodies [Felder et al., 2010]. In acutely ill pigs, this misdirected immune reaction leads to immune-mediated immune-haemolytic anaemia connected with strong *M. suis* reduction in the blood [Jungling et al., 1994; Zachary and Smith, 1985]. Furthermore, activation of intravasal clotting and subsequently disseminated intravascular coagulation (DIC) were observed leading to increased bleeding tendency and overall to disturbance of haemostasis [Plank and Heinritzi, 1990]. As a possible result, micro thrombi and acrocyanoses may occur. This haemostasis disorder has been discussed to be caused directly by clotting-induced endothelial damage or indirectly by present hypoglycaemic-hypoxic-acidotic metabolic status which is caused by acute *M. suis* infections [Plank and Heinritzi, 1990]. During an acute attack the blood glucose consumption increases strongly leading to a life-threatening hypoglycaemia which is clearly due to metabolic activity

and to intense multiplication of *M. suis* (Figure 4) [Nonaka et al., 1996; Heinritzi et al., 1990b; Smith et al., 1990; Zachary and Smith, 1985]. The acidosis results from an increase in the lactic acid concentration as well as from a disturbance in the pulmonary gas exchange [Heinritzi et al, 1990; Smith et al., 1990; Henry, 1979].

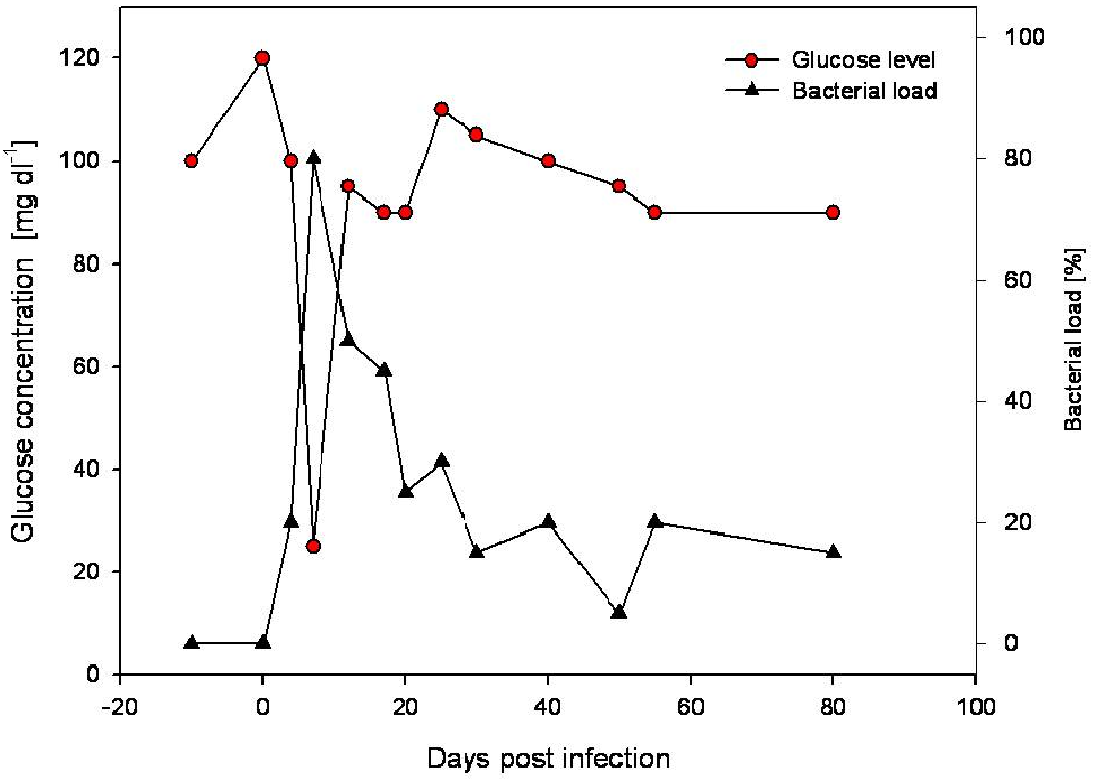

Figure 4. Hypoglycaemia is one typical symptom of acute IAP. As shown during an experimental *M. suis*-infection, blood glucose suddenly decreases in *M. suis*-diseased pigs in direct correlation to rapid multiplication of *M. suis*. Figure according to [Zachary and Smith, 1985].

Beside the auto reactive immune response mentioned above, an *M. suis*-specific immune response was found. Recently, a detailed analysis of the humoral immune response during experimental infection resulted in the identification of at least eight *M. suis* specific antigens which are targeted by specific IgG antibodies [Hoelzle et al., 2006]. Three proteins, i.e. p40, p45, and p70 were the preferentially recognised *M. suis* antigens. This IgG immune response was observed in all infected pigs by 14 days post infection at the latest and until week 14, the end of the experiments. A serological proteome study subsequently performed revealed six immunogenic

M. suis antigens which are expressed during acute clinical *M. suis* infections: two heat shock proteins (DnaK, GroEL), two glycolysis-proteins (enolase, pyruvate dehydrogenase complex), an RNA helicase, and an actin-analogous protein [Hoelzle et al., 2007a]. Shotgun sequencing and screening of genomic *M. suis* DNA libraries enabled the gene identification of two immunodominant *M. suis* proteins: the above DnaK-like heat shock protein HspA-1 (p70) and MSG1 (*M. suis* glycolytic protein; p40) with high similarity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). MSG1 is found as glycolytically active protein in the cytosol, as well as adhesion protein on the cell surface [Hoelzle et al., 2007b]. HspA-1 is also expressed in the *M. suis* membrane indicating its potential participation in adhesion to the erythrocyte [Hoelzle et al., 2007a]. Both in *E. coli* recombinant expressed proteins were recognised by convalescent sera of *M. suis*-infected animals and, therefore, used to establish an ELISA test for serological diagnostics [Hoelzle et al., 2007e]. An additional protein i.e. the soluble inorganic pyrophosphatase (sPPase) was found by said *M. suis* library screening [Hoelzle et al., 2010]. Most peculiar, *M. suis* sPPase is a metal-dependent enzyme (Mg^{2+}) that shows its highest enzyme activity at pH 9.0, and is inhibited in the presence of Ca^{2+} and EDTA. In *M. suis,* the sPPase builts tetramers of 80 kDa which were detected by convalescent sera from experimentally *M. suis*-infected pigs [Hoelzle et al., 2010].

3.2.7 *In vitro* cultivation of *M. suis*

Until now, *M. suis* is still regarded as uncultivable *in vitro*. Thus, research relies on the propagation and maintenance of *M. suis* in splenectomised piglets by subsequent artificial infection [Hoelzle et al., 2003; Zachary and Basgall, 1985; Heinritzi, 1984]. Peripheral anti-coagulated blood samples from pigs were collected at the time point of maximum bacteraemia. EDTA or Alsever´s solution was used as anti-coagulants. The *M. suis* load in infected blood was confirmed by microscopic examination of acridine orange-stained blood smears and quantified by *M. suis*-specific quantitative real-time Light Cycler-PCR (qLC-PCR) based on the *msg*1-gene of *M. suis* [Hoelzle et al., 2007c].

To the best of our knowledge, *in vitro* propagation of *M. suis* or other members of the HM is scarcely described. Only one study dealing with the cultivation of *M. suis* demonstrated a short-term *in vitro* maintenance in a petri dish erythrocyte culture system [Nonaka et al., 1996].

4. Objectives of the Thesis

Mycoplasma suis belongs to the group of highly specialised haemotrophic mycoplasmas (HM) which possess a unique lifestyle on the surface of erythrocytes. HM are uncultivable so far. All attempts to cultivate these fastidious bacteria *in vitro* using cell-free media as well as RBC cultures have failed so far. This reflects the high degree of adaptation to the host environment in HM. A complex interplay of the host environment in connection with RBCs provides the bacteria with the essential nutrients which are necessary for HM multiplication. Furthermore, as all mycoplasmas, *M. suis* has a significantly reduced genome. Therefore it could be assumed that multifunctional proteins occur. In accordance with these facts this Ph.D. thesis deals with three main issues: which nutrients are indispensible for *in vitro* propagation? How does *M. suis* generate ATP and what transport capacities are available for the complex interchange with the erythrocytes? Has the glycolytic enzyme alpha-(α)-enolase a second function beyond their well-characterised metabolic function (as a so called moonlight protein)?

4.1 In vitro *propagation of* M. suis

In vitro cultivation of HMs would replace all animal experiments which are currently necessary for the multiplication of these agents. Cultivation of bacteria is an important feature of microbiology since Robert Koch. Substantial amounts of pure bacteria are (i) the precondition for analyses of bacterial virulence and pathogenesis and (ii) the basis for the implementation of prophylactic measures since vaccine development and production is only possible from culture-derived HMs. Culture systems will allow attenuation and genetic manipulations of HM strains.
Within this thesis *M. suis* cultivation was approached by inoculating different *Mycoplasma* media with anti-coagulated blood from experimentally *M. suis*-infected and acutely diseased pigs, and by stepwise diversifying proved culture techniques for mycoplasmas.

4.2 Analysis of energy metabolism and transport pathways of M. suis

The lifecycle of HMs is dependent on their intimate contact with the host erythrocytes. During an experimental *M. suis* infection, severe hypoglycaemia occurs in direct correlation to the parasitaemia and to the glucose consumption of the agent. Therefore, the analysis of energy metabolism and transport pathways of *M. suis* that harbour potential pathogenic and virulent traits, is a central topic of this Ph.D. thesis.

4.3 M. suis *alpha-enolase as virulence factor*

Although certain housekeeping enzymes, in particular some glycolytic enzymes, do not have classical signal sequences for membrane location; they are displayed on the cell surface ready to interact with the host cell. A further main focus of this doctoral thesis is the identification and functional characterisation of the alpha (α)-enolase protein of *M. suis* in respect to immunogenicity, surface accessibility and potential adhesive properties.

Within the dissertation presented, these three subprojects were consecutively described in following sections. Two paper manuscripts were added to the corresponding sections (5.4; 7.1).

5. *In vitro* Propagation of *M. suis*

The major drawback for HM research was and still is the *in vitro* uncultivability. Until now, all attempts to grow HM in cell-free media have failed and the propagation of e.g. *M. suis* in a splenectomised pig model is the only way for the investigation of the physiology and virulence-determining factors of *M. suis*.

Many other mycoplasmas can be successfully cultured *in vitro* in standard liquid *Mycoplasma* medium. Based on the recently established phylogenetic classification of HM within the genus *Mycoplasma* [Neimark et al., 2001], we hypothesise that HM could be propagated *in vitro* by applying and diversifying proved culture techniques for mycoplasmas. In doing so, it was crucial to provide the microorganisms with the appropriate components from their natural host environment, i.e. in case of *M. suis* from the porcine blood.

In this study two different standard *Mycoplasma* media i.e. *Spiroplasma* SP-4 medium (ATCC medium no. 988) and a modified Hayflick medium [Hayflick, 1965] were initially chosen for the *in vitro* cultivation approaches. Anti-coagulated blood from experimentally *M. suis*-infected pigs was used as inoculum. A quantitative real-time Light Cycler-PCR assay based on the *msg*1-gene of *M. suis* was used to determine and to quantify the *M. suis* growth [Hoelzle et al, 2007c]. Based on the results of the first cultivation approach the SP-4 medium was modified with the best-suited additives, and new cultures were incubated in different cultivation systems (liquid culture systems, solid systems (agar plates) as well as combined systems (liquid and solid media in one tube). Some parts of these culture experiments performed are presented in the **paper manuscript 5.4**. In this manuscript we assumed that iron and/or glucose may be major limiting factors for *M. suis* cultivation. Therefore, the SP-4 *Mycoplasma/Spiroplasma* medium was modified with selected iron-carrier blood components i.e. haemin, haemoglobin or transferrin and compared with a glucose-enriched modified Hayflick *Mycoplasma* medium. Furthermore, the ultrastructure of the *M. suis* cultures grown on agar plates and the *in vitro* interaction of culture-derived *M. suis* with porcine erythrocytes were studied by scanning electron microscopy (SEM).

5.1 Material and Methods

Animal experiments and blood collection

Three five-week-old piglets were splenectomised and experimentally infected with *M. suis* as described earlier [Hoelzle et al., 2003; Heinritzi, 1984]. Peripheral anti-coagulated blood was taken at the time of maximum bacteraemia. Na-citrate was used as anti-coagulant. *M. suis* bacteraemia was microscopically confirmed by acridine orange-staining of blood smears and quantified by *M. suis*-specific quantitative real-time Light Cycler-PCR (qLC-PCR) [Hoelzle et al., 2007c]. Collected blood was used as inoculum within 8 h or stored at -80°C until used.

Liquid media used for *M. suis* cultivation

A standard *Mycoplasma/Spiroplasma* medium (SP-4; ATCC medium 988) was prepared using PPLO broth base (Becton Dickinson, Basel, Switzerland), tryptone (Becton Dickinson), 0.5% glucose (Sigma), 0.5x CMRL-1066 (Gibco/Invitrogen, Basel, Switzerland), 0.35x yeast extract (Gibco), 1x yeastolate (Gibco), and penicillin G (final concentration (f. c.) 1000 U/ml; Sigma). Then, 17% heat-inactivated (56°C, 1h) porcine serum (obtained from the slaughterhouse) or fetal calf serum (FCS; Oxoid) were added under sterile conditions. The medium was further substituted individually or in combination with various porcine blood components, i.e. 5% erythrocyte lysate (EC lysate), 10 mg/l transferrin, 10 mg/l haemoglobin and/or 1% embryonal extract (Figure 5).

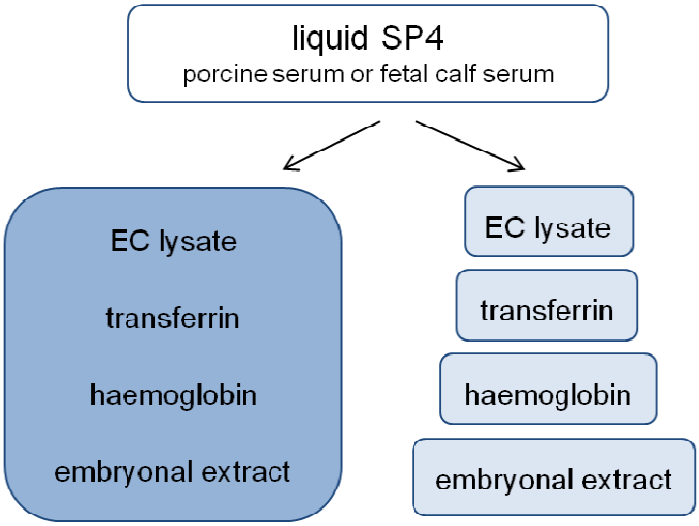

Figure 5. liquid SP-4 medium substituted with different porcine blood additives. EC lysate, porcine erythrocyte lysate.

Porcine embryonal extract. Porcine foetuses were collected and stored at -20°C until processing. Foetal organs (heart, lung, liver, and kidney) were excised, homogenised with mortar and sea sand, and suspended in phosphate-buffered saline (PBS). After low-speed centrifugation (200 x g, 5 min) the supernatant was sterile-filtered (0.2 µm), and added to the SP-4 medium as described above.

Porcine erythrocyte lysate. M. suis-negative anti-coagulated blood (in Alsever´s solution, 60 ml) was washed three times in PBS and centrifuged (2000 x g, 30 min). Erythrocyte pellet was mixed with 30 ml ice-cold lysis buffer (Na-phosphate buffer (5mM, pH 7.6): 4.35 mM Na_2HPO_4, 0.65 mM NaH_2PO_4; 1 mM EDTA), incubated 5 min at room temperature (RT) and stored at -20°C until used. Before use the lysate was thawed and treated by ultrasonication (1 min) on ice and centrifuged for 30 min at 4°C (20000 x g). The pellet was resuspended in 3 ml PBS, and sterile-filtered.

Porcine iron-binding proteins. Stock solutions of 0.1% haemoglobin and transferrin were prepared in PBS, sterile-filtered and added to the medium at a final concentration (f. c.) of 10 mg/l. Haemoglobin was purchased from Sigma, transferrin from First Link (U.K.), LTD.

Solid media used for *M. suis* cultivation

SP-4-agar was prepared as described above (SP-4 liquid medium) by using PPLO agar base (Becton Dickinson) instead of PPLO broth base. Modified Hayflick agar plates were prepared using PPLO agar base and 30% *Mycoplasma* enrichment mixture (Becton Dickinson) of horse serum, yeast extract and thallium acetate. Finally, both agars were substituted with 0.5% glucose (Sigma; Figure 6).

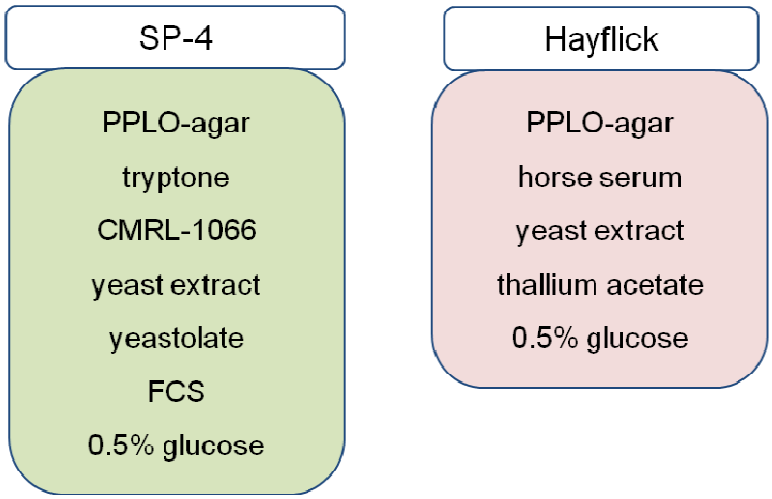

Figure 6. Composition of two *Mycoplasma* agars used in this study: standard SP-4 (ATCC medium 988) and modified Hayflick according to [Hayflick, 1965].

In vitro-cultivation of *M. suis* using different culture systems

M. suis cultures were grown in liquid, solid and combined systems. Liquid cultures were performed using cell culture flasks (25 ml volume) or shell vial tubes. Solid media were performed using small petri dishes (Ø 6 cm). The combined system consisted of an agar-containing plastic carrier which was floated by liquid medium (Figure 7).

Figure 7. Different *in vitro* culture systems.

M. suis culture

All cultures were performed in a 10% CO_2 atmosphere at 37°C. Cultivation approaches were divided into two parts: in a first step liquid cultures were performed, and in a second step subcultures from the liquid cultures were done on agar plates. Cultivation time of liquid cultures started on day 0, with the addition of the quantified

M. suis inoculum (f. c. 5x 10^4 *M. suis*/ml medium). Samples were taken on days 7 and 14 post inoculation (p. i.) and then at two-week intervals for 16 weeks. Samples were used to extract bacterial DNA. DNA was subjected to qLC-PCR in order to measure *M. suis* growth. Cultures were subcultured monthly on solid agar plates using standard bacterial loops. Growth on agar plates was controlled weekly by microscopic examination of the agar plates.

PCR techniques

DNA was extracted from culture samples by using the GenElute Bacterial Genomic DNA Kit (Sigma). *M. suis* load was quantified by applying a quantitative Light Cycler PCR (qLC-PCR) assay based on the *msg*1-gene of *M. suis* as described earlier [Hoelzle et al., 2007c]. Additionally, to further specify the bacterial growth on Hayflick agar, a 16S rDNA PCR was performed according to [Hoelzle et al., 2011]. For this, *M. suis* subcultures were completely scraped off from an inoculated Hayflick agar plate, collected in PBS and subjected to DNA extraction. By using HM-specific 16 S rDNA primers (16S_haemo_forw: GGCCCATATTCCT(AG)CGGGAAG; 16S_haemo_rev: AC(AG)GGATTACTAGTGATTCCA) and HotStarTaq Polymerase (Qiagen) a partial HM 16S rDNA gene fragment was amplified and sequenced by 4 baselab GmbH (Reutlingen, Germany). The obtained gene sequence was compared to databank entries using the BLASTn and FastA algorithms (Biocomputing, University of Zurich; http://www.bio.uzh.ch/).

5.2 Results

***M. suis*-cultivation using SP-4 medium containing different porcine blood derivatives**

Standard *Mycoplasma* medium SP-4 was supplemented with porcine erythrocyte lysate, transferrin, haemoglobin and/or embryonal extract and inoculated (f.c. 5 x 10^4 *M. suis*/ml). Samples were taken on day 7 and 14, and then in a 14 days interval. *M. suis* was quantified by qLC-PCR. Overall, no continuous *M. suis* growth was achieved with any medium used. Nevertheless, differences between the media used were observed (Table 1): Using the media containing SP4 + embryonal extract alone and SP4 + all four additives (EC lysate, transferrin, haemoglobin, and embryonal extract) no *M. suis* was detected 3 weeks after inoculation. The PCR results

remained negative until the end of the experiment. In all other culture approaches using different SP-4 medium compositions (Table 1) a significant decrease in the *M. suis* loads was observed on day 7 and 14. From week 4 onwards the *M. suis* contents of the cultures remained on a stationary level (from 10^0 to 10^2 *M. suis*/ml medium) until the end of the culture periods (16 weeks post inoculation).

Table 1. *M.suis*-cultivation using SP-4 with different substitutions.

Medium	Supplements	*M. suis* PCR results
SP-4	porcine serum	+
SP-4	fetal calf serum	+
SP-4	FCS + EC lysate	+
SP-4	FCS + transferrin	+
SP-4	FCS + haemoglobin	+
SP-4	FCS + embryonal extract	-
SP-4	FCS + all four additives	-

culture conditions: 10% CO_2, 37°C; FCS, fetal calf serum; EC lysate, erythrocyte lysate

M. suis-cultivation using different culture systems

Based on the first results described above, the optimised medium (SP-4 containing FCS, transferrin, haemoglobin and glucose) was used to perform culture experiments in several liquid, solid and combined systems (liquid and solid media in one tube) (Table 2). Liquid and combined culture systems were inoculated with (f.c.) 5 x 10^4 *M. suis*/ml medium. The agar plates were inoculated with 10 µl blood of an experimentally *M. suis*-infected pig.

All *M. suis* liquid cultures (i.e. in shell vial tubes (filled with 3 ml liquid SP-4) and in cell culture flasks) and the combined systems (containing Hayflick agar and liquid SP-4 medium) revealed *M. suis* loads at low levels (over a time period of 16 weeks. However, the *M. suis* loads did not increase in any system used and no *M. suis* propagation was detected.

On the agar plates inoculated with *M. suis* no bacterial growth was detected by light microscopy. However, the 16S rDNA PCR analysis of samples taken from inoculated plates revealed positive results. The 16S rDNA PCR products were 99.9% identical to the 16S rDNA of *M. suis* (strain 3804; GenBank: FN984917).

Table 2. *M. suis*-cultivation using liquid, solid and combined culture systems.

Culture systems	Medium	*M. suis* PCR results
cell culture flasks	SP-4	+[1]
shell vial tubes	SP-4	+[1]
combined systems (liquid/solid)	Hayflick-agar + SP-4	+[1]
petri dishes	Hayflick-agar	+[2]

culture conditions: 10% CO_2, 37°C; SP-4 optimised with fetal calf serum, transferrin, haemoglobin, and glucose; +[1], positive qLC-PCR; +[2], positive 16S rDNA-PCR

5.3 Conclusions

The present thesis aimed at establishing a suitable *in vitro* cultivation system for *M. suis.* Cultivation was attempted by stepwise diversifying a well-known *Mycoplasma* medium (SP-4) with several host-specific supplements. The initial hypothesis was that *M. suis* might need host-specific blood derivatives for its *in vitro* growth. Different sera, i.e. porcine serum and FCS, were tested as undefined rich blood additives. A further suggestion was that *M. suis* growth may be dependent on nutrients derived from porcine erythrocytes since so far replication of *M. suis* has solely been demonstrated on the surface of erythrocytes [Hoelzle, 2008; Zachary and Basgall, 1985]. Therefore, the media used were supplemented with porcine erythrocyte lysate. In addition, it was considered that iron-supplying blood components might be necessary for *M. suis* growth. Due to the peculiar environment of HMs and its close association with their host cells i.e. erythrocytes special iron acquisition systems could play an important role in the establishment of *in vitro* cultivation systems. Other haemotrophic bacteria i.e. *Bartonella* require blood, erythrocytes or haemin for *in vitro* propagation [Carroll et al., 2000]. Actually,

Bartonella quintana, the agent of trench fever, has the highest reported *in vitro* haemin requirement for any bacterium [Carroll et al., 2000]. Therefore, the cultivation approach contained different iron-binding proteins present in blood, i.e. transferrin and haemoglobin.

Overall, none of the culture approaches used resulted in the propagation of *M. suis* as it is known for other mycoplasmas. However, it is not possible to propagate all known mycoplasmas. In terms of their growth behaviour *in vitro* we can generally distinguish three different groups of mycoplasmas. A large group including *M. hominis*, *M. arginini*, *M. mycoides*, *M. capricolum*, *M. laidlawii*, *M. pneumoniae* or spiroplasmas, respectively, is fastidious concerning its nutritional requirements, but it can be grown slowly *in vitr*o within the time period ranging from several days to three weeks [Pereyre et al., 2009; Suthers et al., 2009; Voros et al., 2009; Lin et al., 2008; Hackett et al., 1987; Lee et al., 1983; Tully et al., 1979; Rodwell et al., 1969b; Tourtellotte et al., 1964]. Other *Mycoplasma* species, i.e. *M. genitalium* or *M. penetrans*, are extremely difficult to isolate by culture [Jensen, 2004; Röske et al., 2001; Fraser et al., 1995]. It takes several weeks until colonies appear on *Mycoplasma* agar plates. Moreover, until now only six different *M. penetrans* isolates obtained from clinical samples have been grown in pure culture [Röske et al., 2001]. Finally, there are mycoplasmas which have resisted all attempts of cultivation in cell-free media, e.g. haemotrophic mycoplasmas and phytoplasmas, indicating that they have a different metabolism compared to the other mycoplasmas [Gasparich, 2010; Hoelzle et al., 2008; Kube et al., 2008].

Although no continuous *M. suis* growth took place in the different media used, we were capable of maintaining *M. suis* on a kind of stationary level over a period of 16 weeks (i.e. maintenance). Such *in vitro* maintenance for *M. suis* was not possible in each medium composition but in SP-4 media which were supplemented either with porcine or fetal calf serum combined with erythrocyte lysate, transferrin, or haemoglobin. Between the porcine and fetal calf serum no significant differences were seen. Therefore, FCS was used as serum supplement for further cultivation experiments since sterile FCS could be easily purchased. Furthermore, no differences were measured in cultures with or without erythrocyte lysate. Therefore, we assumed that erythrocytic membrane components are not the crucial step for the *M. suis* cultivation. Then, it was claimed that *M. suis* might require multi-potent cells in combination with erythrocytes. In fact, supplementation with porcine embryonal

extract had rather negative effects on growth. Both the addition of transferrin and haemoglobin clearly had a positive effect on *M. suis* growth. Finally, the supposition that *M. suis* might only be able to grow primarily on solid surface before getting in liquid culture has not been confirmed. We found no continuous growth in the combined system used.

Based on these findings it was concluded that iron and/or glucose might be a major limiting factor to grow *M. suis* in pure culture. Therefore, the SP-4 medium was optimised with three different Fe-supplying blood components, i.e. haemin, haemoglobin or transferrin, respectively, at a ten-fold higher concentration [f. c. 100 µg/ml] than in previous approaches used. Alternatively, we performed cultivation in a simplified modified Hayflick medium enriched with 1% glucose [Gardella et al., 1995; Hayflick, 1965]. Detailed description of media used and results obtained from these two new culture approaches are given in the **paper manuscript 5.4**.

5.4 Paper manuscript

In vitro cultivation of the haemotrophic *Mycoplasma suis* in cell free media induces nanotransformation

Sabrina A. Schreiner, Katharina Hoelzle, Regina Hofmann-Lehmann, Anja Hamburger, Max M. Wittenbrink, Manuela M. Kramer, Albina Sokoli, Kathrin M. Felder, Katrin Groebel, Ludwig E. Hoelzle

Manuscript submitted

Until now, no *in vitro* system has been developed for continuous *M. suis* growth. Nevertheless, our approach was successful in maintaining *M. suis* cells in pure culture over a short time-span. Additionally, we analysed the ultra-structural features of *M. suis* grown on *Mycoplasma*-agar, and investigated the infectivity of culture-derived *M. suis* cells by means of scanning electron microscopy.

Own contributions:
I designed the study and defined the strategies for cultivation. Further, I prepared all media, performed inoculation and sampling of cultures, analysed the results for optimisation of the culture conditions and wrote the manuscript.

In vitro cultivation of the haemotrophic *Mycoplasma suis* in cell free media induces nanotransformation

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Abstract

Background. *Mycoplasma suis* belongs to the haemotrophic mycoplasmas (HMs) which cause infectious anaemia in a large variety of vertebrates. So far, no *in vitro* cultivation system for the propagation of *M. suis* and other HMs has been established. We hypothesised that *M. suis* could be grown in classical *Mycoplasma* media supplemented with appropriate nutrients (e.g. glucose, iron-binding proteins) available in their natural environment, i.e. in the porcine blood.

Findings. In this study, anti-coagulated blood from experimentally *M. suis*-infected pigs was used to inoculate either the standard *Mycoplasma* medium SP-4 substituted with iron-binding proteins, or the glucose-enriched Hayflick *Mycoplasma* medium. A quantitative *M. suis*-specific real-time PCR assay was used to determine and quantify the *M. suis* loads at the time point of inoculation and during cultivation. One week after inoculation, the *M. suis* loads in the cultures decreased remarkably and then remained at a stationary level over the time span of twelve weeks in all medium compositions used, thus indicating a kind of maintenance. Scanning electron microscopic analysis of *M. suis* cells subcultivated on agar plates revealed small microcolonies in dense clusters. Within these microcolonies irregular single *M. suis* cells of reduced size (approximately 0.2 to 0.6 µm) were observed indicating a transformation of *M. suis* into special life forms that are referred to as nanoforms. Sequence analysis of the partial 16S rDNA of the cultured *M. suis* cells revealed nearly 100% identity with the 16S rDNA of *M. suis* deposited in GenBank. Culture-derived *M. suis* cells were able to infect porcine erythrocytes *in vitro*. We found single *M. suis* organisms attached to the erythrocyte by fibril-like structures.

Conclusions. The cultivation of *M. suis* in modified standard *Mycoplasma* media is obviously not appropriate for its propagation, but leads to a kind of maintenance of *M. suis* outside the host. Furthermore, the adaptation of *M. suis* to conditions unfavourable for growth was accompanied by alteration into special morphological structures known as nanoforms. The found nanoforms seemed to retain infectivity *in vitro*.

Findings

Background

Mycoplasma (M.) suis is a member of the haemotrophic mycoplasmas (HMs) which attach to the surface of host erythrocytes or invade their target cells [1, 2]. HMs are found in a wide range of animals, e.g. cats, dogs, mice, cattle, sheep, swine, horses, llamas, sea lions, and primates [1, 3, 4, 5]. In all these animals HMs are considered host-specific and to cause infections clinically characterised either by an overt life-threatening haemolytic anaemia or by a subtle chronic anaemia, by ill thrift, infertility, and immune suppression leading to higher susceptibility to infections with other bacteria. Furthermore, it is evident that HM infections are also apparent in humans [6, 7, 8, 9, 10].

HMs are highly specialised bacteria with a high degree of host adaptation reflected by its specific cell tropism, persistent infection, immune modulation, and, finally, uncultivability *in vitro*. The unique lifestyle of HMs is dependent on their intimate contact with their host erythrocytes. In the case of *M. suis,* fine fibrils which are obviously responsible for connection were observed on the attachment site between the agent and the erythrocyte membrane [4, 11]. Furthermore, close interaction between *M. suis* and its host cell is accompanied by severe deformation of the erythrocytic surface with prominent pits, trenches and invaginations.

Until now no *in vitro* cultivation system for any HM member has been established. All attempts to cultivate these fastidious organisms *in vitro* using cell-free media as well as erythrocyte cultures have failed so far [1, 4]. Only a short-term maintenance of *M. suis* has been achieved so far, using a petri dish erythrocyte culture system [12]. Therefore, to date, HM research relies on the propagation in splenectomised animals by animal-to-animal passages, a method connected with serious ethical concerns and with host cell contaminations of bacterial cells [13]. The establishment of an *in vitro* cultivation system would replace all these animal experiments. Moreover, cultivation of bacterial organisms is an important feature of microbiology since Robert Koch. Substantial amounts of pure bacteria are the precondition for the analyses of bacterial characteristics and would enable the development of strategies for therapy and prophylaxis of HM infections.

In this study we hypothesised that HMs could be grown in pure culture by applying and diversifying proved culture techniques for mycoplasmas. To specifically

determine and quantify the *M. suis* growth, we used a quantitative real-time Light Cycler-PCR assay based on the *msg*1-gene of *M. suis* [14]. Furthermore, we investigated the ultrastructure of the *M. suis* by scanning electron microscopy (SEM) after subcultivation on agar plates, and studied the *in vitro* interaction of culture-derived *M. suis* cells with porcine erythrocyte cultures.

Material and Methods

Animal experiments and blood collection

To produce the *M. suis* inoculum piglets were splenectomised and experimentally infected with *M. suis* as described earlier [15, 16]. Blood was drawn in sterile vacutainer tubes containing Na-citrate as anti-coagulant at maximum bacteraemia as confirmed by microscopic examination of acridine orange-stained peripheral blood smears and by *M. suis*-specific quantitative real-time Light Cycler-PCR [14].

For *in vitro* infection studies of erythrocytes, EDTA anti-coagulated blood from *M. suis*-negative pigs was used. Blood was centrifuged (500 x g, 15 min) and erythrocytes were washed three times in phosphate-buffered saline (PBS; Sigma, Buchs, Switzerland). The leukocyte-rich buffy coat was removed after the first centrifugation step. Finally, the erythrocyte sediment was suspended in RPMI-1640 medium (pH 7.4; Sigma) containing 10% fetal bovine serum (FBS; Oxoid, Basel, Switzerland).

Media used for *M. suis* cultivation

A standard *Mycoplasma*/*Spiroplasma* medium (SP-4, ATCC medium 988; pH 7.0) was prepared using PPLO broth base (Becton Dickinson, Basel, Switzerland), tryptone (Becton Dickinson), 0.5% glucose (Sigma), 0.5x CMRL-1066 (Gibco/Invitrogen, Basel, Switzerland), 0.35x yeast extract (Gibco), 1x yeastolate (Gibco), 17% heat-inactivated fetal calf serum (FCS; Oxoid), and penicillin G (final concentration (f. c.) 1000 U/ml; Sigma). Porcine haemin (Sigma), haemoglobin (Sigma) or transferrin (First Link LTD; U.K.) was diluted in phosphate-buffered saline (PBS), sterile-filtered (0.2 µm), and added individually (f. c. 100 mg/l).

The modified Hayflick *Mycoplasma* medium [17] was prepared using PPLO broth base and 30% *Mycoplasma* enrichment mixture (Becton Dickinson) of horse serum, yeast extract and thallium acetate. Finally, 1% glucose and penicillin G (f. c. 1000

U/ml) were added. For the Hayflick agar plates the PPLO broth base was replaced by PPLO agar base.

M. suis culture

All *M. suis* liquid cultures were performed in shell vial tubes (containing 3 ml medium) in a 10% CO_2 atmosphere at 37°C. Cultivation time started on day 0 with the addition of 30 µl blood from *M. suis*-infected pigs containing 1.5 x 10^7 *M. suis*/ml. Samples were taken at day 0 and then weekly, and subjected to DNA extraction and quantitative real-time Light Cycler-PCR. Subcultures on Hayflick agar plates were carried out from the liquid cultures after 4, 8 and 12 weeks of incubation.

Infection of porcine erythrocytes with cultured *M. suis*

For the erythrocyte infection assays cells were suspended in RPMI containing 10% FCS (2.5% erythrocytes), and mixed in a 1:1 ratio with an *M. suis*-positive liquid culture. Negative control erythrocytes were mixed with medium alone. Erythrocytes were incubated in 1.5 ml reaction tubes (Eppendorf, Basel, Switzerland) at 37°C for five days.

Quantitative Light Cycler-PCR (qLC-PCR)

M. suis liquid cultures (3 ml) were centrifuged (20000 x g, 20 min), and the pellet was resuspended in 200 µl PBS. Bacterial DNA was extracted by using the GenElute Bacterial Genomic DNA Kit (Sigma). To determine the *M. suis* loads of the culture samples a quantitative Light Cycler-PCR assay was performed as described previously [14].

16S rDNA-PCR and sequencing

To specify the growth on Hayflick agar plates a partial 16S rDNA PCR fragment was amplified as described earlier [18]. Thus, *M. suis* subcultures were completely scraped off a Hayflick agar plate, collected in PBS and subjected to DNA extraction. The resulting PCR amplicon was sequenced (4 baselab, Reutlingen, Germany) and the resulting gene sequences were compared with GenBank entries using the FastA algorithm (http://www.bio.uzh.ch/)

Scanning electron microscopy (SEM)

M. suis subcultures on Hayflick agar plates (8 weeks p.i.) were fixed with 3% PBS-buffered glutaraldehyde (GA) solution for 1 h. Small agar pieces (0.5 cm in diameter) were cut out and incubated in 3% GA solution overnight. After washing (3x) in distilled water, fixed agar samples were post-fixed in 1% osmium tetroxide (Fluka Chemie, Buchs, Switzerland). After dehydration using increasing concentrations of ethanol (25-100%), and critical point drying (BAL-TEC CPD 030 critical point dryer; Balzers, Liechtenstein) the samples were sputter-coated with 12 nm Pt/C particles using the BAL-TEC MED 020 coating system (Balzers), and analysed with a Zeiss Supra 50 VP scanning electron microscope.

Infected and non-infected erythrocytes were fixed with 2.5% GA and settled on 10 nm carbon-coated cover slips using a Cytospin 2 (Shandon, Dako-Diagnostica, Zug, Switzerland) centrifuge. The post-fixation of samples in 2% osmium tetroxide (Fluka Chemie, Buchs, Switzerland) was followed by subsequent dehydration using increasing concentrations of acetone (25-100%) and critical point drying. Samples were then further prepared and analysed by SEM as described above.

Results

M. suis-cultivation using SP-4 medium containing different iron-containing blood components

To date, iron-acquisition systems used by HMs are unknown. We hypothesised that iron which is naturally found in the host's blood bound to haemin, haemoglobin or transferrin, could be one major limiting factor for the growth of HMs in pure culture. Therefore, the classical *Mycoplasma* medium SP-4 was supplemented with haemin, haemoglobin or transferrin, respectively, and inoculated with blood from experimentally infected pigs. The inoculated cultures had a final *M. suis* concentration of 5 x 10^4*M. suis*/ml. Samples were taken weekly. The results of the qLC-PCR quantification of the culture aliquots taken weekly are shown in Figure 1. After inoculation (p.i.) a decrease in the *M. suis* loads from mean 1.1 x 10^4*M. suis*/ml medium (week 0) to mean 4.02 x 10^2 (1 week p.i.), and mean 1.45 x 10^2 (week 2 p.i.) *M. suis*/ml medium was observed. From week 2 onwards the *M. suis* contents of the cultures remained on a stationary level of about 1 to 5 x 10^2 *M. suis*/ml medium

(mean value) until the end of the experiment (week 12 p.i.). No significant differences in the *M. suis* culture loads were found between the different media approaches.

M. suis-cultivation using glucose-enriched modified Hayflick medium

Previous *in vitro* studies with *M. suis*-infected blood revealed a high *M. suis* glucose consumption with a maximum of 144 mg/dl/h [19] leading to the assumption that *M. suis* uses glucose as its main energy source. Therefore, we used a modified Hayflick medium enriched with 1% glucose [17, 20] for the cultivation of *M. suis*. Inoculation, incubation and culture load determination (qLC-PCR) were performed as described above. The *M. suis* culture loads decreased rapidly from mean 8.5 x 10^3 on average (inoculation) to mean 9.4 x 10^2 (week 1 p.i.), and mean 1.3 x 10^2 (week 2 p.i.). From week 2 onwards the *M. suis* contents of the cultures remained on a stationary level of 0.9 to 2.1 x 10^2 *M. suis*/ml medium (mean value) until the end of the experiment (week 12 p.i.).

Scanning electron microscopic analysis of *M. suis* cultures

M. suis cells derived from liquid Hayflick cultures were subcultured on Hayflick agar plates. Eight weeks p.i. the ultrastructure of cultured *M. suis* cells was studied by SEM. We found *M. suis* microcolonies of varied size of between 2 x 4 µm to 2 x 10 µm distributed over the whole agar plate surface (Figure 3). Within the microcolonies, single *M. suis* organisms with a small cell size ranging between 200 to 600 nm in diameter appeared to be closely connected to and barely distinguishable from each other. The *M. suis* cells observed demonstrated irregular and round shapes. The HM-specific 16S rDNA PCR sequence analysis of the *in vitro* subcultures revealed 99.9% identity to the 16S rDNA sequence of *M. suis* (strain 3804; GenBank: FN984917).

In vitro infection of porcine erythrocyte cultures with culture-derived *M. suis*

Erythrocytes from a non-infected *M. suis*-negative pig were inoculated in a 1:1 ratio with a liquid eight-week-old *M. suis* culture. After five days of incubation the ultra-structural properties of the *M. suis* interaction with the erythrocytes were analysed by SEM. We found erythrocytes infected with single *M. suis* cells. As shown in Figure 4, the connection between erythrocyte and *M. suis* organisms seemed to be mediated by fibril-like extensions.

Discussion

Despite a long known significance of haemotrophic mycoplasmas no *in vitro* cultivation system has been established so far [1, 21]. To the best of our knowledge, to date, no systematic experimental studies concerning HM propagation in cell-free media have been published. In the present study we attempted to propagate *M. suis in vitro* based initially on two working hypotheses:

First, we claimed that HM cultivation in standard *Mycoplasma* medium could be successful if iron-containing proteins i.e. haemin, haemoglobin or transferrin which could be physiologically found in blood were added. Iron is frequently known to be the limiting factor for bacterial growth. Other haemotrophic bacteria such as *Bartonella* are known to require erythrocytes or haemin supplements in order to grow *in vitro*. In fact, *B. quintana* has the highest known haemin requirement for a bacterium [22, 23]. Overall, using iron-supplemented SP-4 medium we were capable of maintaining *M. suis* on a kind of stationary level over a period of 12 weeks (i.e. maintenance) but we did not achieve noticeable growth. In contrast, previous culture experiments using other *Mycoplasma* media i.e. SP-4 medium or PPLO broth without iron-containing proteins revealed completely negative results at least after 4 weeks of incubation (data not shown). However, the *Mycoplasma* medium used in this study resulted in a kind of maintenance, but was obviously not suitable for a continuous growth of *M. suis*, and the substitution of iron-containing proteins was not the sole critical step for *M. suis* cultivation. Furthermore, it is not possible now to render any statement as to which iron-containing protein could be used by *M. suis* for iron acquisition, as we found no significant differences between the media used. However, recent analysis of the whole *M. suis* genome sequences revealed that haemin would be the most probable iron-supplying protein, since, similar to *Bartonella*, *M. suis* possesses a haemin-specific transporter [24, 25].

The second approach is based on the fact that life-threatening hypoglycaemia is one major complication of an acute *M. suis*-infection [1]. The metabolism of *M. suis* is obviously dependent on glucose and the metabolic activity of *M. suis* is the most logical reason for the dramatic decrease of the glucose level in the blood [12, 19]. Furthermore, during maturation, porcine erythrocytes lose their permeability for glucose, and do not utilise glucose as the primary energy source [26].These facts have now been further supported by the results of the *M. suis* whole genome

sequencing indicating that *M. suis* generates ATP solely through glycolysis [24]. We therefore used a modified Hayflick medium enriched with 1% glucose which corresponds approximately to tenfold the physiological glucose concentration in the porcine blood (3.3 to 6.8 mM/l or 0.05 to 0.15% glucose concentration) [17, 20, 27]. Cultivation in this medium also revealed a kind of maintenance similar to the first approach: After a remarkable decrease of the *M. suis* culture load in the first two weeks of incubation, a nearly constant *M. suis* culture load could be observed until the end of the experiment. Therefore, it can be assumed that the liquid glucose-enriched Hayflick medium was also not suitable to continuously propagate *M. suis in vitro*.

In the next step we found cultured *M. suis* cells building microcolonies after subcultivation on agar plates. Analyses of the ultrastructure by SEM revealed strong evidence that our cultivation approach induces a transformation of *M. suis* into nanoforms that are ultra microforms (nanotransformation). Nanocell formation has already been described for other mycoplasmas, i.e. *Acholeplasma laidlawii* and *M. gallisepticum* [28, 29]. We found densely packed *M. suis* microcolonies of small variable sizes of between 2 x 4 µm to 2 x 10 µm on the inoculated Hayflick agar plates leading to the assumption that the *M. suis* detected were obviously not inoculum residues. This was further supported by the fact that no erythrocyte residues could be found in connection with the cultured *M. suis* cells. The morphology of the culture-derived *M. suis* cells within the microcolonies differed distinctly from erythrocyte-associated *M. suis* cells in size and shape: The *M. suis* nanoforms were irregular and more compact roundish. In *Acholeplasma laidlawii* and *M. gallisepticum,* nanotransformation could be induced by cultivation in adverse growth conditions (e.g. deficient medium or temperature shift) as an adaptive response. In addition to the morphological changes, *A. laidlawii* and *M. gallisepticum* nanoforms differed significantly from its vegetative forms in their protein profiles [28, 29]. Obviously, the media used are suitable for a kind of propagation (maintenance) but provided no optimal growth conditions for *M. suis* which is reflected by said nanotransformation. Interestingly, *M. suis* derived from liquid cultures were able to infect healthy porcine erythrocytes *in vitro* indicating viability of the maintained *M. suis* cells.

Conclusions

In this study, we were able, for the first time, to demonstrate a special form of propagation of the haemotrophic *M. suis* outside the host. However, the media used are not appropriate for a continuous *M. suis* growth, but rather provide *M. suis* adverse growth conditions leading to a transformation into nanoforms. In the future, the analysis of the whole genome of *M. suis* sequenced recently [24] will help to identify the essential medium components for the establishment of *in vitro* cultivation systems for the propagation of HMs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SAS performed the cultivation assays, sample collection and data acquisition, wrote the manuscript; AS, KMF and KG performed sample preparation and SEM; MMK and AH performed the DNA extraction of culture samples and the qLC-PCR assay; KH performed the animal experiments, helped with the experimental study design and drafted the manuscript; RHL and MMW helped with the design of the study; LH designed, planned and coordinated the study, supervised the experiments and the manuscript. All authors read and approved the final manuscript.

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Figures

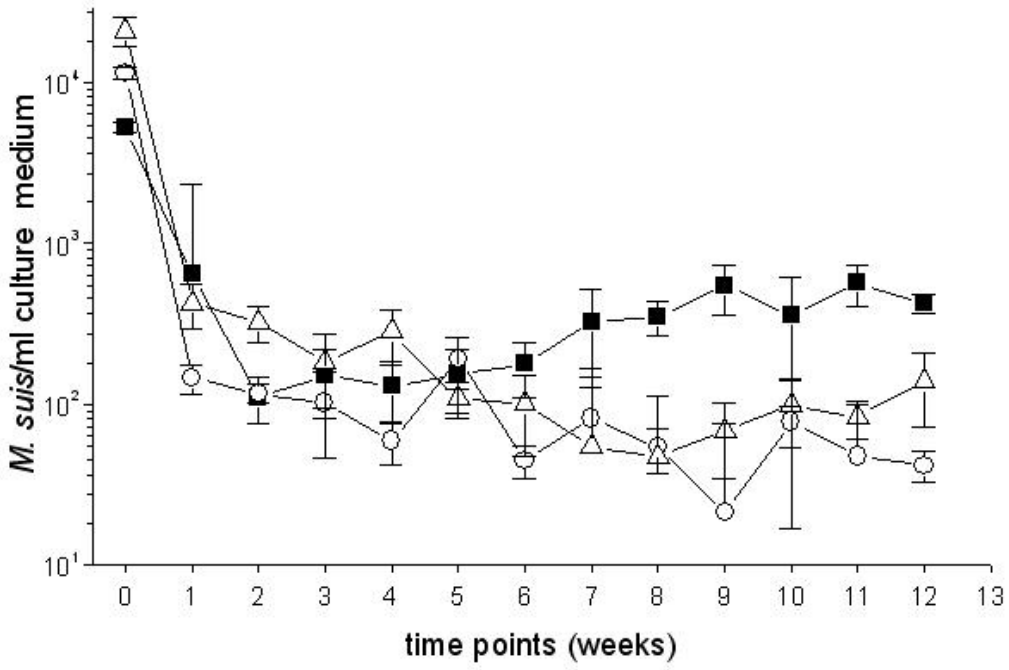

Figure 1

***M. suis* propagation in SP-4 *Mycoplasma* medium.** SP-4 medium (ATCC medium 988) supplemented with haemin (○), haemoglobin (Δ), or transferrin (■) was inoculated using blood from an experimentally *M. suis* infected pig (f.c. 5 x 10^4 *M. suis*/ml medium). Samples were taken weekly for 12 weeks. The *M. suis* loads were determined by a quantitative LC-PCR [14].

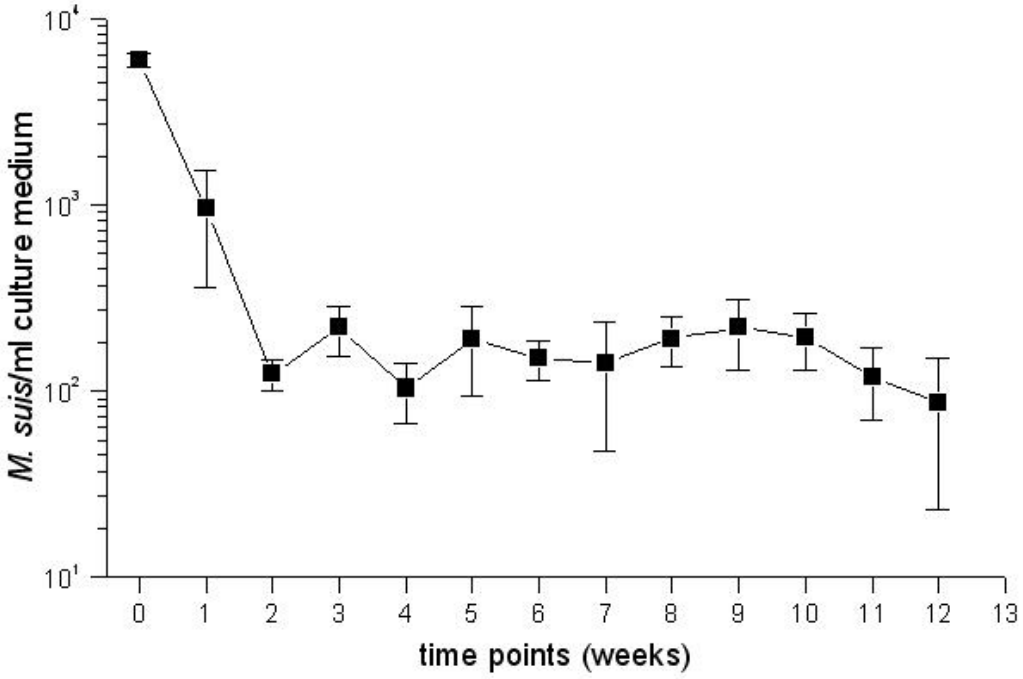

Figure 2

M. suis **propagation in glucose-enriched Hayflick medium.** Hayflick medium supplemented with 1% glucose (■) was inoculated using blood from an experimentally *M. suis* infected pig (f.c. 5 x 10^4 *M. suis*/ml medium). Samples were taken weekly for 12 weeks. The *M. suis* loads were determined by a quantitative LC-PCR [14].

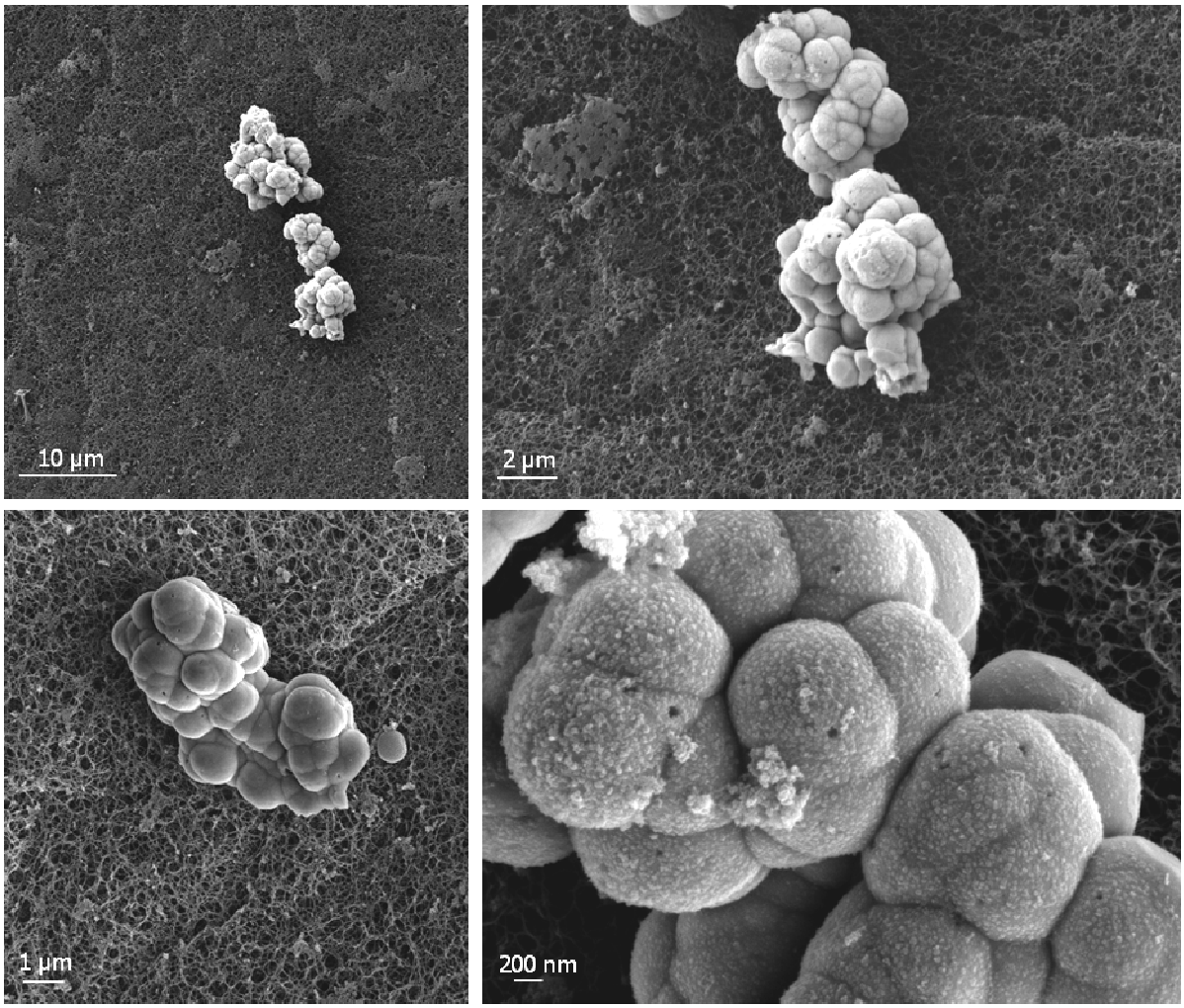

Figure 3

Scanning electron microscopy (SEM) of *M. suis* cultures grown on Hayflick-agar. *M. suis* cultures were subcultivated on Hayflick agar plates. Eight weeks p. i. agar pieces were fixed and analysed using SEM. *M. suis* microcolonies with varied size between 2 x 4 µm to 2 x 10 µm could be found on the agar surface. Within the microcolonies irregular roundish small *M. suis* cells ranging between 200 to 600 nm in diameter could be distinguished.

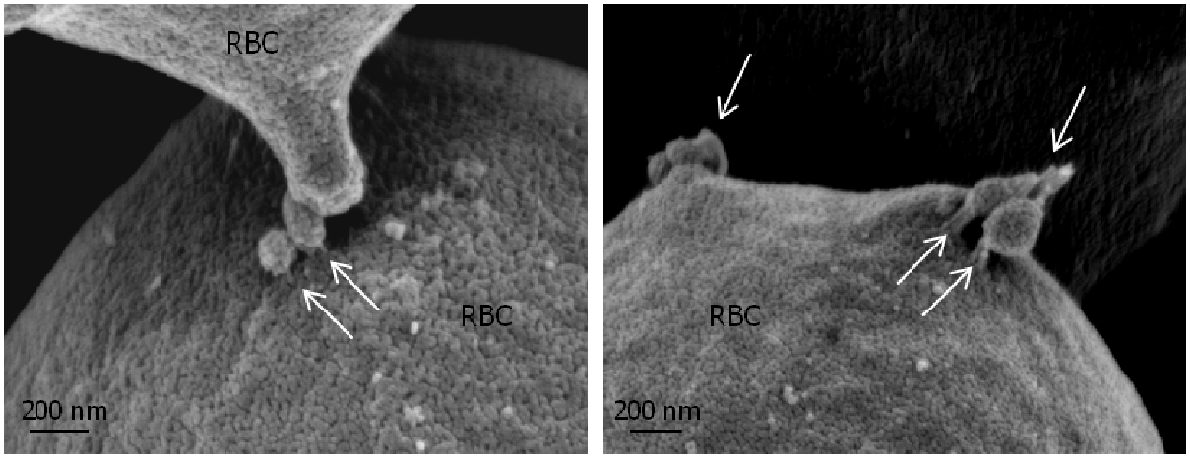

Figure 4

Scanning electron microscopy (SEM) of porcine erythrocytes infected with culture-derived *M. suis*. Porcine erythrocytes were incubated with *M. suis*-positive liquid cultures. After incubation for five days *M. suis* cells (arrows) attached to the surface of the red blood cells (RBCs) could be observed. Fibril-like extensions seem to mediate the contact between erythrocyte and *M. suis* organisms.

6. Analysis of Energy Metabolism and Transport Pathways of *M. suis*

One common property of all *Mycoplasma* species is their dependency on the metabolism of eukaryotic cells leading to an intimate connection to mostly epithelial or endothelial cells [Pitcher and Nicholas, 2005; Razin et al., 1998; Nicolet, 1996]. In contrast, haemotrophic mycoplasmas are adapted to a parasitic lifestyle on the host's red blood cells. This lifestyle is unusual for mycoplasmas and even all bacteria, and there is strong evidence that the metabolism of these haemotrophic bacteria has significantly diverged from other mycoplasmas (e.g. *M. genitalium*, *M. pneumoniae*) as well as from other well-studied bacteria. Since mature RBCs are non-proliferative, enucleated and lack mitochondria their metabolism is mainly dependent on glycolysis to produce ATP [Climent et al., 2009].

Until the beginning of 2011, no comprehensive sequence data of *M. suis* were available and very little was known about the biology and about virulence factors of all haemotrophic *Mycoplasma* species. Due to the impossibility of culturing them, the definition and characterisation of the metabolism and pathogenic features was very difficult and the knowledge was mainly based on few empirical studies. Several experimental studies in piglets provided evidence of glucose consumption by metabolic activity of *M. suis* itself. As a result, severe, often life-threatening hypoglycaemia occurs during an acute clinical "attack" [Nonaka et al., 1996; Heinritzi et al., 1990b; Smith et al., 1990; Zachary and Smith, 1985]. This observation leads to the assumption that glucose metabolism of *M. suis* might play a central role in its lifecycle.

In this work genomic shotgun libraries of *M. suis* were sequenced. Subsequent Southern blot hybridisation and *in silico* DNA analyses were used to reconstruct metabolic pathways and transport systems in order to gain insight into the lifestyle of these rather unexplored bacteria. One advantage of these methods is that only low amounts of *M. suis* are necessary. However, since the *M. suis* DNA used for the construction of the libraries was purified from experimentally infected pigs, a huge background of porcine DNA had complicated the work. Despite the encountered difficulties, I was able to characterise and reconstruct the sugar uptake system and the glycolysis pathway of *M. suis* that are probably competing with the host cell´s

metabolism. Furthermore, I could identify some transport systems which seem essential for viability and virulence of several pathogens, i.e. the Sec-translocase machinery and the phosphate-specific ABC-transporter Pst. In the future, the characterisation of the biological features and metabolic characteristics of *M. suis* will be the basis for the development of culture strategies and for the definition of the metabolism as well as of the role of *M. suis* in causing illness.

6.1 Material and Methods

Purification of *M. suis* cells

M. suis-infected blood was obtained from experimentally infected animals at maximum bacteraemia after *M. suis* confirmation by microscopic examination of acridine orange-stained blood smears and by *M. suis*-specific quantitative real-time Light Cycler-PCR [Hoelzle et al., 2007c]. Whole blood was collected in Alsever's solution (Sigma, Buchs, Switzerland) as anticoagulant. Blood cells were sedimented by low-speed centrifugation (300 x g, 15 min). Plasma and buffy coat were discarded. The sediment consisting of *M. suis*-laden erythrocytes was suspended in phosphate-buffered saline (PBS, pH 7.4; Sigma) containing 0.15% Tween-20 and 3% EDTA and incubated (20 min, at room temperature (RT) with gentle agitation to separate *M. suis* cells from erythrocytes. Debris and erythrocytes were removed by centrifugation (500 x g, 20 min). *M. suis* cells were sedimented by high-speed centrifugation (45000x g, 2 h, 4°C). The resulting *M. suis* pellet was suspended in 2 ml PBS and stored at -80°C until used.

Library construction and sequence analysis

M. suis DNA was purified from *M. suis* cell suspensions as described elsewhere [Hoelzle et al., 2003]. Library construction was performed by Medigenomics (Martinsried, Germany). Briefly, *M. suis* DNA fragments averaging about 1.5 kb (Ms_library1) and 2.5 to 3.0 kb (Ms_library2) were ligated into a blunt-end cut, *Sma*I-digested pUC19 vector. In order to control the quality of the two libraries and to detect *M. suis* sequences involved in pathogenic processes 600 randomly selected clones were sequenced (MWG, Ebersberg, Germany). Nucleotide sequences were analysed using the BLASTn/BLASTx and the FastA algorithm (Biocomputing, University Zurich, www.bio.unizh.ch) to compare the specific *M. suis* clones to

sequences from other mycoplasmas deposited in GenBank. For determination of putative open reading frames (ORFs) we used an ORFfinder program (www.ncbi.nlm.nih.gov/projects/gorf/). Translation of ORFs to amino acid sequences was performed by taking into account the alternative genetic codon usage of mollicutes (UGA as tryptophan codon (UGA_{Trp}) instead of a stop codon).

Southern Blot hybridisation of genomic *M. suis* DNA

To complete fragmentary *M. suis* gene sequences of interest obtained from the shotgun library clones, Southern blot analyses were subsequently carried out. Briefly, genomic *M. suis* DNA was digested with different restriction enzymes (e. g. *Eco*RI, *Hind*III) and separated by agarose gel electrophoresis. After capillary blotting and DNA-transfer onto nylon membranes, restricted *M. suis* DNA was incubated with several digoxigenin (DIG)-labelled probes derived from *M. suis* shotgun insert sequences. Detected DNA fragments were cloned in a pUC19 vector (Roche, Rotkreuz, Switzerland), transformed into *E. coli* Top10 and fully sequenced (MWG).

6.2 Results

6.2.1 Sequence analyses of the *M. suis* shotgun libraries

Sequences from genomic *M. suis* shotgun library clones (n=600) were analysed. A total of 78 clones definitively contained *M. suis* DNA, the remaining clones consisted of porcine DNA impurities.

All clones containing gene sequences with high homologies (best hit; % identity in nt overlap) to known genes of other related mycoplasmas are shown in Table 3. *M. suis* genes and gene fragments involved in the energy metabolism, sugar uptake, and adhesion were identified. Furthermore, genes encoding for transport proteins, DNA-binding proteins, and proteins with function in the transcriptional and translational processes, as well as ribosomal proteins, were found in the shotgun library. As shown in Table 4, some corresponding proteins were confirmed in context with a concomitant Ph.D. proteomic study using liquid chromatography coupled to mass spectrometry [Felder K. M., dissertation, 2010]. The *M. suis* genes found in the shotgun library were compared to the whole genome sequence of *M. suis* (strain KI_3806; GenBank: FQ790233) published in January 2011 by [Oehlerking et al., 2011]. All *M. suis* library genes were also present in the whole genome sequence of

M. suis. Annotated open reading frames (ORFs) of the *M. suis* whole genome were signed by the locus_tag of each predicted gene (Table 4).

Table 3. Cross species identification of *M. suis* coding gene sequences by genomic shotgun screening and subsequent Southern blot hybridisation of genomic *M. suis* DNA. Putative *M. suis* genes were determined comparing databank (NBCI) entries of other related *Mycoplasma* species (see GenBank).

Gene	Function/Protein	Clone	Highest Homology	GenBank	Best Hit (% identity in nt overlap)
	Adhesion				
*a*1	Heat-shock protein (70)	SG1-F1	*M. suis*	AM265536	99.0 % identity in 784 nt
	Adhesion, Glycolysis				
*msg*1	GAPDH-like protein	SG1-H10 SG1-76 SG2-92	*M. suis*	AM407404	97.2 % identity in 788 nt 96.4 % identity in 390 nt 99.5 % identity in 387 nt
pgk	Phospho-glycerokinase	SG1-H10 SG1-76 SG2-92	*M. penetrans*	AP004174	63.1 % identity in 665 nt 59.3 % identity in 703 nt 68.7 % identity in 365 nt
*pfk*A	Phospho-fructokinase	SG1-66	*M. capricolum*	CP000123	60.3 % identity in 585 nt
eno	Enolase	SG1-66	*M. capricolum*	CP000123	62.5 % identity in 934 nt
pgm	Phospho-glyceromutase	SG2-156	*M. mobile*	AE017308	60.3 % identity in 784 nt
	Glucose Uptake				
*pts*I	Enzyme I	SG3-D9	*M. gallisepticum*	AE016967	65.2 % identity in 353 nt
*pts*H	Phosphocarrier (HPr)	SG2-148 SC-195	*U. parvum*	AE002119	56.7 % identity in 264 nt 51.5 % identity in 240 nt
*hpr*K	HPr-kinase/phosphatase	SG1-76 HPrK-10	*M. penetrans*	AP004174	56.8 % identity in 714 nt 58.4 % identity in 824 nt

Gene	Function/Protein	Clone	Highest Homology	GenBank	Best Hit (% identity in nt overlap)
	Transporter				
*pst*A	Phosphate ABC-transporter permease	PstB-32	*M. gallisepticum*	AE016967	53.9 % identity in 312 nt
*pst*B	Phosphate ABC-transporter ATPase	SG2-H1 SC-195 PstB-15 PstB-32	*U. parvum*	AE002119	63.3 % identity in 294 nt 53.4 % identity in 861 nt 59.3 % identity in 403 nt 68.7 % identity in 565 nt
*sec*A	Membrane pre-protein translocase	SG2-E10 SG1-146 SG3-18	*M. gallisepticum*	AE016967	56.8 % identity in 952 nt 57.0 % identity in 964 nt 60.3 % identity in 1080 nt
ffh	Signal recognition particle (SRP)	SG1-90 SG4-7	*M. gallisepticum*	AE015450	62.3 % identity in 202 nt 65.0 % identity in 199 nt
*fts*Y	SRP-receptor (FtsY)	HPrK-10	*M. capricolum*	CP000123	59.3 % identity in 465 nt
*oxa*A	Membrane protein translocase (YidC)	SG3-38	*M. pneumoniae*	U00089	58.7% identity in 513 nt
aap/*pot*E	Putative amino acid permease	SG2-H1	*M. pulmonis*	AL445564	56.3 % identity in 1515 nt
*opp*F	Oligoendopeptidase F	SG2-11	*M. penetrans*	BA000026	54.8 % identity in 628 nt
*trk*G	Potassium (K^+) transporter	SG2-G3	*M. mycoides*	BX842644	53.4 % identity in 470 nt
*mgt*E	Magnesium (Mg^{2+}) ion transporter	SG1-53	*M. pulmonis*	AL445564	57.0 % identity in 526 nt
	DNA-Binding Proteins				
*rpo*D	RNA-polymerase D (Sigma factor 70)	SG4-7	*M. pneumoniae*	U00089	66.7 % identity in 807 nt
*pol*A	DNA polymerase (5´-3´- exonuclease)	SG2-152 SG2-A7	*M. mycoides*	BX293980	62.2 % identity in 232 nt 61.5 % identity in 405 nt
*rpo*B	DNA-directed RNA-polymerase B	SG2-50 SG3-B5 SG2-E10 SG2-116	*M. penetrans*	AP004170	65.4 % identity in 765 nt 65.1 % identity in 269 nt 63.9 % identity in 814 nt 65.2 % identity in 807 nt
*ruv*A/B	DNA-helicase (subunits A/B)	SG2-B8	*M. penetrans*	AP004171	57.7 % identity in 428 nt
*him*A/B	Histone-like DNA-binding proteins	SG3-D9	*U. parvum*	AE002119	55.2 % identity in 123 nt
sbb	Single-stranded binding protein	SG1-90	*U. parvum*	AE002119	54.6 % identity in 136 nt
*lig*A	NAD-dependent DNA-ligase	SG2-7	*M. penetrans*	BA000026	63.0 % identity in 289 nt
*rec*A	Recombination protein	SG1-165	*M. mycoides*	L22073	59.5 % identity in 778 nt
*hsd*S	Methylase S	SG2-152	*M. pneumoniae*	U00089	52.9 % identity in 606 nt

Gene	Function/Protein	Clone	Highest Homology	GenBank	Best Hit (% identity in nt overlap)
	Transcription/Translation				
*obg*E	GTP-binding protein	SG2-47	*M. mycoides*	BX293980	54.8 % identity in 451 nt
*eng*A	GTP-binding protein	SG3-D9	*M.gallisepticum*	AE015450	56.6 % identity in 778 nt
*prf*A	Peptide-chain release factor 1	SG2-H2 SG2-53	*M. penetrans*	BA000026	58.6 % identity in 760 nt 52.3 % identity in 745 nt
*gre*A	Elongation factor (GreA)	SG2-137	*M. penetrans*	BA000026	62.2 % identity in 135 nt
*fus*A	Elongation factor (EF-G)	SG1-D10	*M. penetrans*	AP004170	69.4 % identity in 673 nt
put. TLF	Predicted translation factor (Sua5-yciO-YrdC)	SG1-120	*M. penetrans*	BA000026	57.4 % identity in 641 nt
*hem*K	Modification methylase	SG2-H2 SG2-53	*M.gallisepticum*	AE015450	49.7 % identity in 316 nt 52.4 % identity in 345 nt
*gua*A	Glutamine-hydrolysing GMP-synthase	SG1-120	*M. pneumoniae*	U00089	58.7 % identity in 826 nt
*gua*B	Inosine-5-monophosphate dehydrogenase	SG1-120	*M. pneumoniae*	U00089	56.1 % identity in 758 nt
rmmbl	RNA-metabolising metallo-ß-lactamase	SG2-H10	*M. pulmonis*	AL445563	51.0 % identity in 225 nt
map	Methionyl-aminopeptidase	SG2-99	*M. pneumoniae*	U00089	59.9 % identity in 452 nt
*gat*A/B/C	Aspartyl/glutamyl-tRNA amidotransferase (ABC)	SG2-34 SG3-31	*M. penetrans* *M.gallisepticum*	BA000026 AE015450	59.5 % identity in 353 nt 59.1 % identity in 342 nt
*nrd*E/F	Ribonucleosid-diphosphate reductase A/B	SG2-104 SG3-27	*M. mycoides*	BX293980	59.1 % identity in 765 nt 57.5 % identity in 777 nt
*nrd*I	Ribonucleoprotein	SG3-27	*M. mycoides*	BX293980	53.3 % identity in 335 nt
*rnp*A	Ribonuclease P	SG3-38 SG2-136	*M. penetrans* *M.gallisepticum*	BA000026 AE015450	56.7 % identity in 421 nt 46.7 % identity in 197 nt
pth	Peptidyl-tRNA-hydrolase	SG2-45	*M. capricolum*	CP000123	57.5 % identity in 461 nt
*met*S	Methionyl-tRNA-synthetase	HPrK-10	*M. penetrans*	AP004170	62.2 % identity in 264 nt
*his*S	Histidyl-tRNA-synthetase	SG3-49	*M. penetrans*	BA000026	56.9 % identity in 757 nt
*ile*S	Isoleucyl-tRNA-synthetase	SG1-172	*M. penetrans*	BA000026	57.3 % identity in 681 nt
*asn*S	Aspariginyl-tRNA-synthetase	SG3-27	*M. mycoides*	BX293980	60.1 % identity in 356 nt
*glt*X	Glutamyl-tRNA-synthetase	SG2-77	*M. penetrans*	BA000026	59.7 % identity in 797 nt
upp	Uracil-phosphoribosyl-transferase	SG2-125	*M. penetrans*	BA000026	58.7 % identity in 605 nt

Gene	Function/Protein	Clone	Highest Homology	GenBank	Best Hit (% identity in nt overlap)
	Metabolism				
*atp*A/B	ATP-synthase A/B	SG3-11 SG2-52	*M. penetrans* *M. pulmonis*	AP004170 AL445563	62.7 % identity in 604 nt 69.4 % identity in 843 nt
lon	ATP-dependent protease La	SG2-D10 SG2-108	*M. penetrans*	AP004172	63.8 % identity in 530 nt 63.4 % identity in 555 nt
*fts*H	ATP-dependent metalloprotease (cell division protein)	SG2-30 SG3-38	*M. penetrans*	BA000026	64.6 % identity in 475 nt 58.7 % identity in 450 nt
adh	Alcohol-dehydrogenase	SG1-21	*M. agalactiae*	CU179680	60.4 % identity in 212 nt
*pur*B	Adenylosuccinate lyase	SG2-C9	*M. penetrans*	AP004172	57.2 % identity in 526 nt
adk	Adenylate-kinase	SG2-99	*M. mycoides*	BX293980	55.9 % identity in 512 nt
*csd*A	Cysteine-desulfurase	SG1-H10	*M. penetrans*	BA000026	54.4 % identity in 525 nt
tdk	Thymidylate-kinase	SG2-11	*M. capricolum*	CP000123	57.5 % identity in 461 nt
*pyr*H	Uridylate-kinase	SG2-57	*M. capricolum*	CP000123	61.9 % identity in 543 nt
	Ribosomal Proteins				
*rps*C	30S ribosomal protein S3	SG1-127	*M. mobile*	AE017308	57.3 % identity in 633 nt
*rps*E	S5	SG2-52	*M. pneumoniae*	U00089	51.5 % identity in 426 nt
*rps*F	S6	SG1-90	*M. penetrans*	BA000026	56.7 % identity in 559 nt
*rps*P	S16	SG2-148	*M. mobile*	AE017308	55.9 % identity in 390 nt
*rps*U	S21	SG2-148	*M. mobile*	AE017308	57.7 % identity in 344 nt
*rpl*A	50S ribosomal protein L1	SG1-36	*M. penetrans*	BA000026	55.3 % identity in 230 nt
*rpl*B	L2	SG1-29 SG1-49	*M. penetrans*	BA000026	67.7 % identity in 713 nt 64.9 % identity in 413 nt
*rpl*F	L6	SG2-52	*M. pneumoniae*	U00089	49.8 % identity in 236 nt
*rpl*K	L11	SG1-36	*M. gallisepticum*	AE015450	65.4 % identity in 647 nt
*rpl*M	L13	SG2-E9 SG2-10	*M. genitalium*	L43967	63.4 % identity in 279 nt 61.6 % identity in 335 nt
*rpl*Q	L17	SG2-148 SC-195	*M. mobile*	AE017308	65.0 % identity in 225 nt 66.8 % identity in 324 nt
*rpl*R	L18	SG2-52	*M. pneumoniae*	U00089	59.2 % identity in 294 nt
*rpl*S	L19	SG2-148	*M. mobile*	AE017308	58.8 % identity in 289 nt
*rpl*T	L20	SG2-9	*M. pulmonis*	AL445563	57.0 % identity in 555 nt

Table 4. Cross species identification of *M. suis* coding gene sequences. The presence of some *M. suis* genes was further supported by a recent Ph.D. proteome study signed by (√) and by the completed annotation of the whole *M. suis* genome (strain KI_3806; GenBank: FQ790233) signed by corresponding ORF numbers (locus_tag) of the predicted genes.

Gene	Functionality	Protein	Locus_tag
	Adhesion		
*a*1	Heat-shock protein (Hsp70)	√	Msui06620
	Adhesion, Glycolysis		
*msg*1	GAPDH-like protein	√	Msui07720
pgk	Phospho-glycerokinase	√	Msui07710
*pfk*A	Phospho-fructokinase	√	Msui06570
eno	Enolase	√	Msui06580
pgm	Phospho-glyceromutase	√	Msui00580
	Glucose Uptake		
*pts*A	Enzyme I	√	Msui04240
*pts*H	Phosphocarrier (HPr)	√	Msui03970
*hpr*K	HPr-kinase/phosphatase	√	Msui07700
	Transporter		
*pst*I	Phosphate ABC-transporter permease		Msui04010
*pst*B	Phosphate ABC-transporter ATPase		Msui04000
*sec*A	Membrane pre-protein translocase		Msui06830
ffh	Signal recognition particle (SRP)		Msui00180
*fts*Y	SRP-receptor (FtsY)	√	Msui07690
*oxa*A	Membrane protein translocase (YidC)		Msui08090
aap/*pot*E	Putative amino acid permease		Msui03990
*opp*F	Oligoendopeptidase F		Msui07650
*trk*G	Potassium (K^+) transporter	√	Msui04150
*mgt*E	Magnesium (Mg^{2+}) ion transporter	√	Msui04030
	DNA-Binding Proteins		
*rpo*D	RNA-polymerase D (Sigma factor 70)		Msui04200
*pol*A	DNA polymerase (5´-3´- exonuclease)	√	Msui02900
*rpo*B	DNA-directed RNA-polymerase B	√	Msui06550
*ruv*A/B	DNA-helicase (subunits A/B)		Msui07360 Msui07370
*him*A/B	Histone-like DNA-binding proteins	√	Msui04280 Msui04290
sbb	Single-stranded binding protein	√	Msui00200
*lig*A	NAD-dependent DNA-ligase	√	Msui02550
*rec*A	Recombination protein	√	Msui07670
*hsd*S	Methylase S		Msui02270

Gene	Functionality	Protein	Locus_tag
	Transcription/Translation		
*obg*E	GTP-binding protein		Msui03080
*eng*A	GTP-binding protein		Msui04300
*prf*A	Peptide-chain release factor 1	√	Msui07960
*gre*A	Elongation factor (GreA)	√	Msui03910
*fus*A	Elongation factor (EF-G)	√	Msui04110
TLF	Putativer translation factor (Sua5-yciO-YrdC)	√	Msui00080
*hem*K	Modification methylase		Msui07950
*gua*A	Glutamine-hydrolysing GMP-synthase	√	Msui04180
*gua*B	Inosine-5-monophosphate dehydrogenase	√	Msui04170
rmmbl	RNA-metabolising metallo-ß-lactamase		Msui04210
map	Methionyl-aminopeptidase	√	Msui03820
*gat*A/B/C	Aspartyl/glutamyl-tRNA amidotransferase (ABC)	√	Msui04250 Msui04260 Msui07330
*nrd*E/F	Ribonucleosid-diphosphate reductase A/B	√	Msui02580 Msui02600
*nrd*I	Ribonucleoprotein		Msui02590
*rnp*A	Ribonuclease P		Msui00010
pth	Peptidyl-tRNA-hydrolase	√	Msui02600
*met*S	Methionyl-tRNA-synthetase	√	Msui07680
*his*S	Histidyl-tRNA-synthetase	√	Msui03100
*ile*S	Isoleucyl-tRNA-synthetase	√	Msui03160
*asn*S	Aspariginyl-tRNA-synthetase	√	Msui02570
*glt*X	Methionyl-tRNA-synthetase	√	Msui03310
upp	Uracil-phosphoribosyl-transferase	√	Msui08010
	Metabolism		
*atp*A/B	ATP-synthase A/B	√	Msui04460 Msui04430
lon	ATP-dependent protease La	√	Msui03540
*fts*H	Cell division protein	√	Msui08060
adh	Alcohol-dehydrogenase		Msui04770
*pur*B	Adenylosuccinate-lyase	√	Msui06630
adk	Adenylate-kinase	√	Msui03790
*csd*A	Cysteine-desulfurase		Msui07730
tdk	Thymidylate-kinase		Msui07660
*pyr*H	Uridylate-kinase	√	Msui04410
	Ribosomal Proteins		
*rps*C	30S ribosomal protein S3		Msui03650
*rps*E	S5		Msui03760
*rps*F	S6	√	Msui00190
*rps*P	S16		Msui03930
*rps*U	S21		Msui03960
*rpl*A	50S ribosomal protein L1	√	Msui02440
*rpl*B	L2		Msui03620
*rpl*F	L6	√	Msui03740
*rpl*K	L11		Msui02430
*rpl*M	L13		Msui04760
*rpl*Q	L17		Msui03980
*rpl*R	L18		Msui03750
*rpl*S	L19		Msui03950
*rpl*T	L20		Msui06790

6.2.2 Analysis of the metabolic and transport pathways of *M. suis*

Sequence analysis of the shotgun library clones revealed proteins connected with the phosphoenolpyruvate:sugar phosphotransferase system (PTS) for the uptake of sugar molecules, with the glycolysis pathway, and with transport systems i.e. Sec-translocase and phosphate-specific ABC (ATP-binding cassette)- transporter (Pst). To further characterise the genetic basis and to identify other components of these important metabolic and transport features, Southern blot analyses of genomic *M. suis* DNA were performed.

6.2.2.1 Sugar uptake via PTS system

The PTS system is mainly responsible for sugar import by concomitant phosphorylation, but it is also implicated in numerous regulatory processes such as up- or down-regulation of carbon catabolite repression/activation (CCR/CCA)-sensitive genes and is found in both gram-positive and gram-negative bacteria (Figure 8) [Deutscher et al., 2005]. It consists of two general soluble components, enzyme I and phosphocarrier HPr, and a membrane-bound enzyme II protein complex. In most gram-positive bacteria, HPr becomes phosphorylated not only at His-15 by PEP and enzyme I but also by ATP-dependent bifunctional HPr-kinase/phosphatase (HPrK/P) at Ser-46. P-Ser-HPr turned out to function as catabolite co-repressor by interacting with the catabolite control protein CcpA [Deutscher et al., 2006; 2005].

In this work the following *M. suis* PTS components were detected in shotgun library clones: (i) HPr in SG2-148; (ii) enzyme I in SG3-D9; and (iii) HPrK/P in SG1-76. The organisation of the genes in the library clones sequenced, is illustrated in Figure 9. The PTS genes found are not clustered together and seem to be rather distributed separately over the *M. suis* genome. A similar distribution of PTS genes can be found when compared to genomes of related mycoplasmas (i.e. *M. genitalium* (GenBank: L43967), *M. pneumoniae* (GenBank: U00089), and *M. penetrans* (GenBank: BA000026). Comparing of the PTS components found with the whole *M. suis* genome sequence revealed complete conformity. Further components of the PTS system i.e. the *crr*-gene and the *pts*G-gene, both parts of the membrane-embedded enzyme II complex (E IIA^{glc} + E $IIBC^{glc}$) were found in the *M. suis* genome indicating

that glycolysis is fed by the uptake of glucose via PTS and the conversion by the glucose 6-phosphate isomerase.

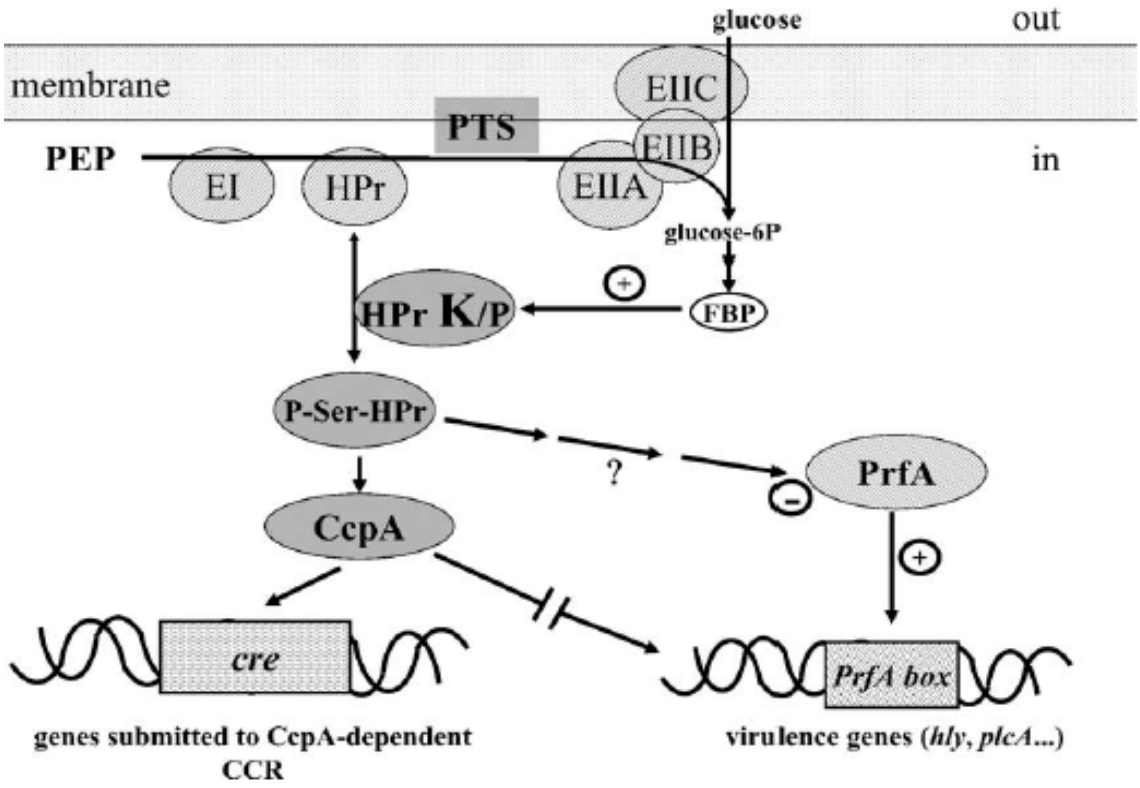

Figure 8. PTS system in gram-positive bacteria (i.e. *Listeria monocytogenes*): HPrK/P acts bifunctional, in response to fructose-bisphosphate (FBP), ATP, and P_i concentrations. P~Ser-HPr interacts via CcpA with *cre*-sites of CCR/CCA-sensitive genes, e. g. genes of some glycolytic enzymes such as phospho-fructokinase (*pfk*), pyruvate kinase (*pyk*) or lactate dehydrogenase (*ldh*). In *L. monocytogenes*, HPr is linked to regulation of virulence genes via Ser-46 phosphorylation. Figure according to [Deutscher et al., 2005].

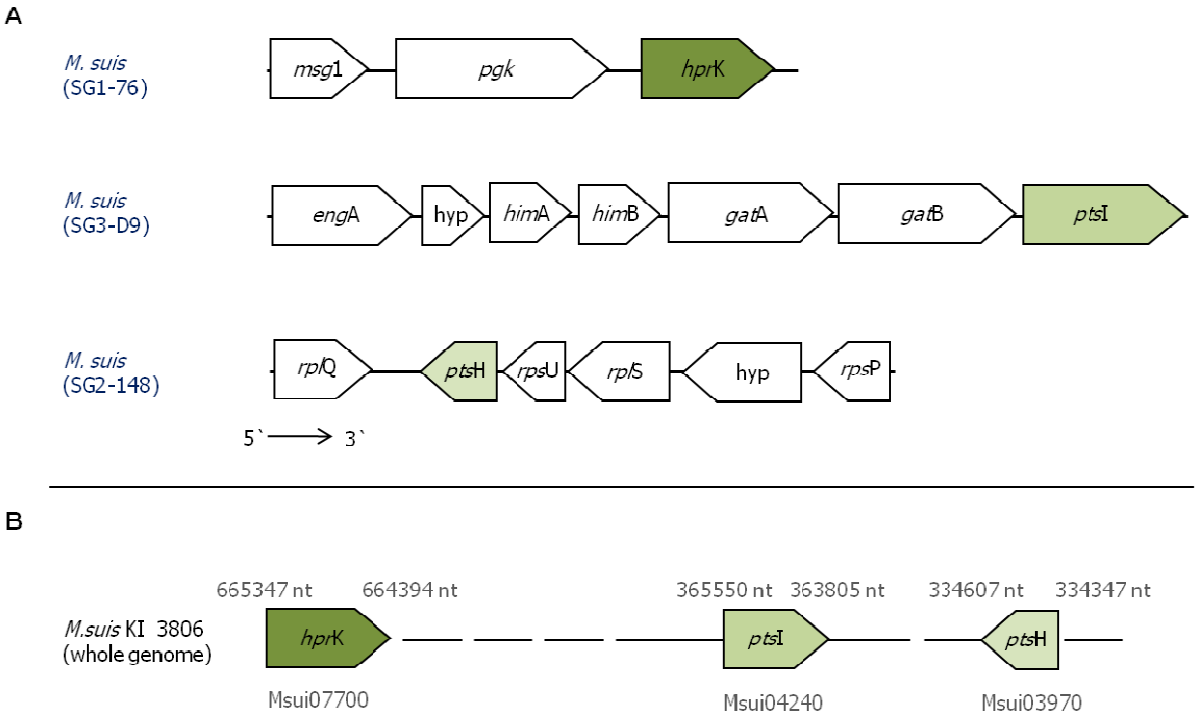

Figure 9. PTS-system: (**A**) Gene organisation of PTS-associated proteins found in *M. suis* shotgun clones (SG1-76, SG3-D9, SG2-148). All detected PTS components are coloured in green: *pts*H, HPr; *pts*I, enzyme I; *hpr*K, HPr-kinase/phosphatase. Surrounding genes are: *msg*1, GAPDH-homolog (*M. suis* glycolytic protein); *pgk*, phospho-glycerokinase; *eng*A, GTP-binding protein; hyp, hypothetical protein; *him*A/B, histone-like DNA-binding proteins; *gat*A/B, aspartyl/glutamyl-tRNA amidotransferase subunits; *rpl*Q, 50S ribosomal protein L17; *rps*U, 30S ribosomal protein S21; *rpl*S, 50S-L19; *rps*P, 30S-S16. (**B**) The same gene organisation was supported by whole genome annotation of *M. suis* strain KI_3806 (GenBank: FQ790233). All PTS genes are separately scattered over the whole *M. suis* genome. The locus_tags and actual nucleotide (nt) positions were placed to the *M. suis* PTS-genes.

6.2.2.2 Glycolysis

Acute IAP is characterised by a radical decrease of the glucose level in the blood of infected pigs. The decreasing blood glucose concentration is clearly associated with the metabolism of *M. suis* [Heinritzi et al., 1990; Smith et al., 1990]. This observation was further supported by the fact that porcine erythrocytes are limited to metabolise glucose due to the lack of functional glucose transporters [Nonaka et al., 1996]. Therefore, the glycolytic pathway of *M. suis* is of special interest.

Several genomic *M. suis* library clones contain gene sequences encoding for proteins of the glycolysis: MSG1 (GAPDH-homolog, SG1-76, SG2-92), PfkA (phospho-fructokinase A; SG1-66), Pgk (phospho-glycerokinase; SG1-76, SG2-92), Pgm (phospho-glyceromutase; SG2-156), and Eno (enolase; SG1-66). Figure 10 illustrated the organisation of the genes found in the sequenced library clones. Indications were found that the glycolytic enzymes of *M. suis* are organised in three clusters. Comparison of the *M. suis* arrangement to related mycoplasmas (i.e. *M. genitalium* (GenBank: L43967), *M. pneumoniae* (GenBank: U00089), and *M. penetrans* (GenBank: BA000026) revealed high similarity (Figure 10). Analysis of the whole genome *of M. suis* demonstrated that the glycolysis pathway is complete, but tricarboxylic acid cycle, arginine hydrolysis, or urea hydrolysis are incomplete or missing. As illustrated in Figure 11, whole genome annotation resulted in the identification of further five glycolytic *M. suis* genes: (i) glucose-6-phosphate isomerase (*gpi*), (ii) fructose-bisphosphate aldolase (*fba*), (iii) triose-phosphate isomerase (*tpi*A), (iiii) pyruvate kinase (*pyk*F), and (iiiii) D-lactate dehydrogenase (*ldh*). The organisation of the glycolytic enzymes in three clusters was confirmed by the *M. suis* genome sequencing. The three clusters consist of (i) TpiA and Pgm; (ii) MSG1 and Pgk; and (iii) Eno, PfkA and Pyk.

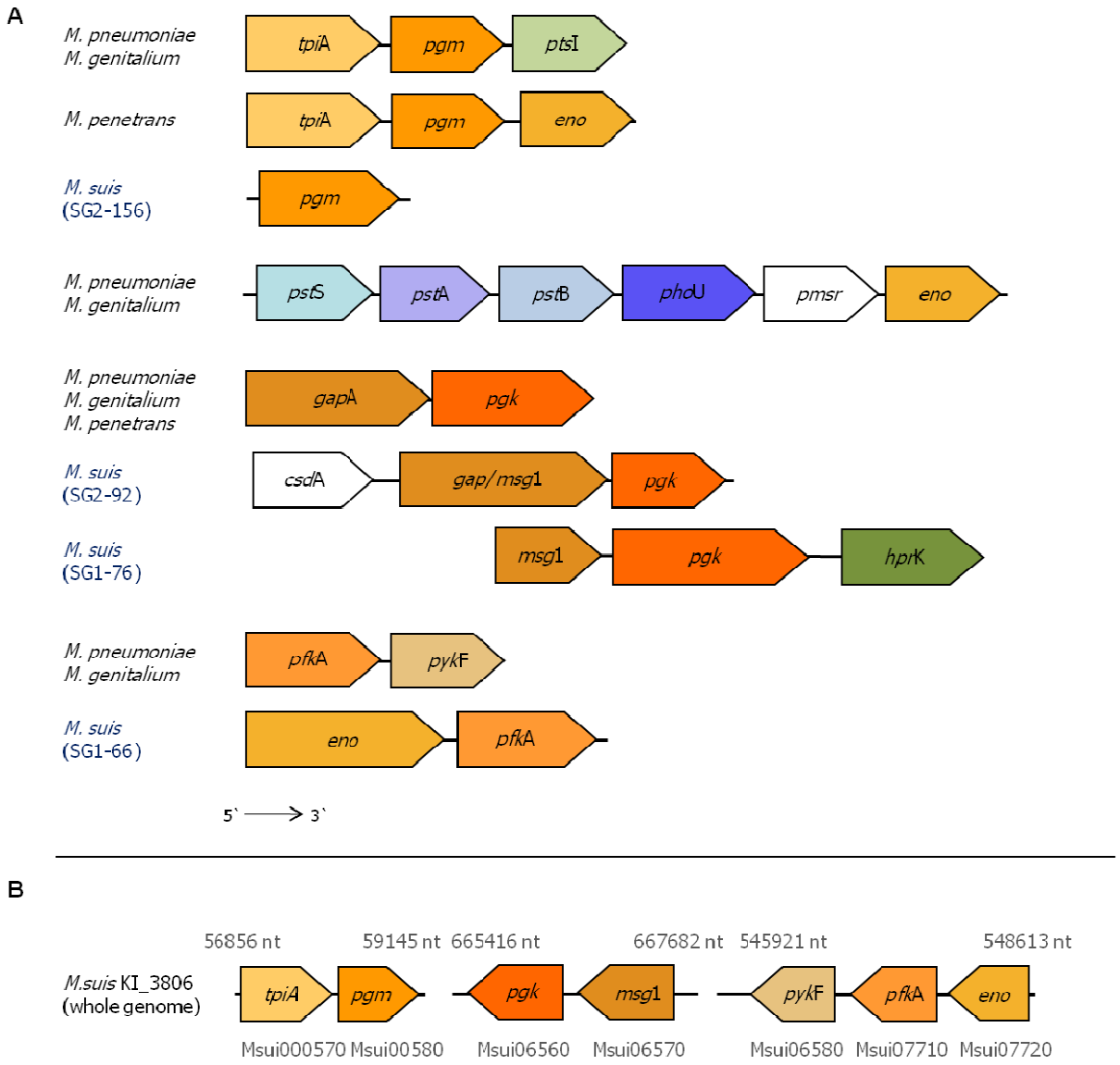

Figure 10. Glycolysis: (**A**) Gene organisation of some glycolytic proteins found in *M. suis* shotgun clones (SG2-156, SG2-92, SG1-76, SG1-66). *M. suis* glycolytic genes are: *pgm*, phospho-glyceromutase; *gap/msg*1, GAPDH (MSG1); *pgk*, phospho-glycerokinase; *eno*, enolase; *pfk*A, phospho-fructokinase A (shown in brownish-orange). PTS genes: *pts*I and *hpr*K (shown in green). Others: *csd*A, cysteine-desulfurase; *pmsr*, methionine sulfoxide reductase A; *pst*SAB-*pho*U, polycistronic operon encoding for phosphate-specific ABC-transporter. (**B**) Whole genome annotation (KI_3806; GenBank: FQ790233) provided evidence of three glycolytic gene clusters in *M. suis* (*tpi*A-*pgm*, *msg*1-*pgk*, and *eno*-*pfk*A-*pyk*F) which are quite similar to gene arrangements in *M. genitalium* (GenBank: L43967), *M. pneumoniae* (GenBank: U00089), and *M. penetrans* (GenBank: BA000026). The locus_tags and actual nucleotide (nt) positions were placed to the *M. suis* glycolytic genes.

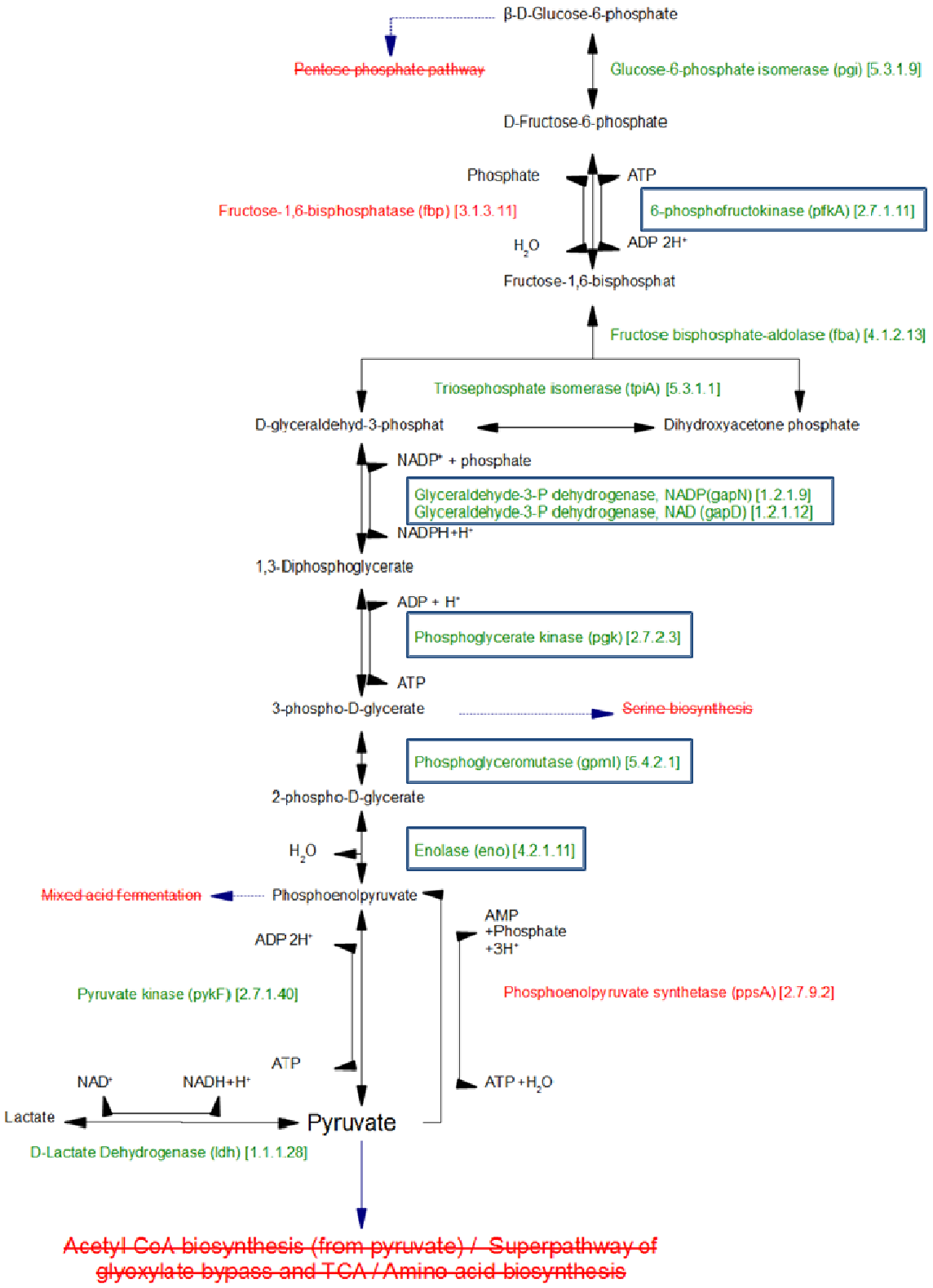

Figure 11. Glycolytic pathways in *M. suis*: Results of shotgun sequencing and whole genome annotation (of *M. suis* strain KI_3806; GenBank: FQ790233) are illustrated. All genes found in the shotgun libraries are bordered in blue. The genes identified by annotation are written in green. All other genes and pathways, respectively, in *M. suis* non-identified are shown in red and/or crossed out in red.

6.2.2.3 Sec-translocase

The Sec-translocase is a major pathway of pre-protein translocation from the cytosol across the cytoplasmic membrane in bacteria. This pathway is also used for integration of some membrane proteins and the Sec-translocase interacts with other cellular components to achieve its cellular roles.

Components of the Sec-machinery are the SecYEG protein complex, a membrane-embedded “channel”, the dimeric SecA and a variety of non-essential ancillary proteins (YidC, SecDF-YajC) [du Plessis et al., 2011; Valent, 1998]. Figure 12 gives an overview of the Sec-translocase found in bacteria. For post-translational protein translocation the mature unfolded pre-protein is captured by the cytosolic chaperone SecB and is driven to the Sec-machinery. The co-translational translocation of both membrane and secretory proteins is targeted by the signal recognition particle (SRP) pathway recognising a signal leader peptide in the ribosome-nascent polypeptide chain. After binding to SRP-receptor FtsY, the nascent protein is transported by the SRP-FtsY complex to the Sec-translocase. The leader sequence is cleaved at the membrane by a signal peptidase releasing the mature protein into the periplasm triggered by SecA (Figure 12) [du Plessis, 2011; de Gier and Luirink, 2003; 2001]. YidC is a relatively abundant inner membrane protein associated with the Sec-translocase or acting as an insertase on its own [Chen et al., 2002; de Gier and Luirink, 2001; Samuelson et al., 2000]. Interestingly, YidC has also been implicated in the insertion of SecE [Yi et al., 2003]. The SecDFyajC domain controls the pre-protein movement by regulating SecA membrane-cycling through the membrane [Duong and Wickner, 1997].

In this work the following *M. suis* components of the Sec-translocase were identified in different clones: SRP (*ffh*; SG1-90), FtsY (*fts*Y; HPrK-10), YidC (*oxa*A; SG3-38) and SecA (*sec*A; SG2-E10). Figure 13 illustrates the results of the shotgun library sequencing and the Southern blot hybridisation. No gene sequences were found for the SecB pathway. Comparison to the published *M.suis* whole genome sequences confirmed the results obtained from the library. Moreover, *M. suis* genome annotation identified homologous genes for the SecY and SecE components of the membrane channel but no genes corresponding to SecG and to the SecDFyajC complex. As a consequence, *M. suis* obviously use the SRP pathway to target pre-proteins to the Sec-translocase.

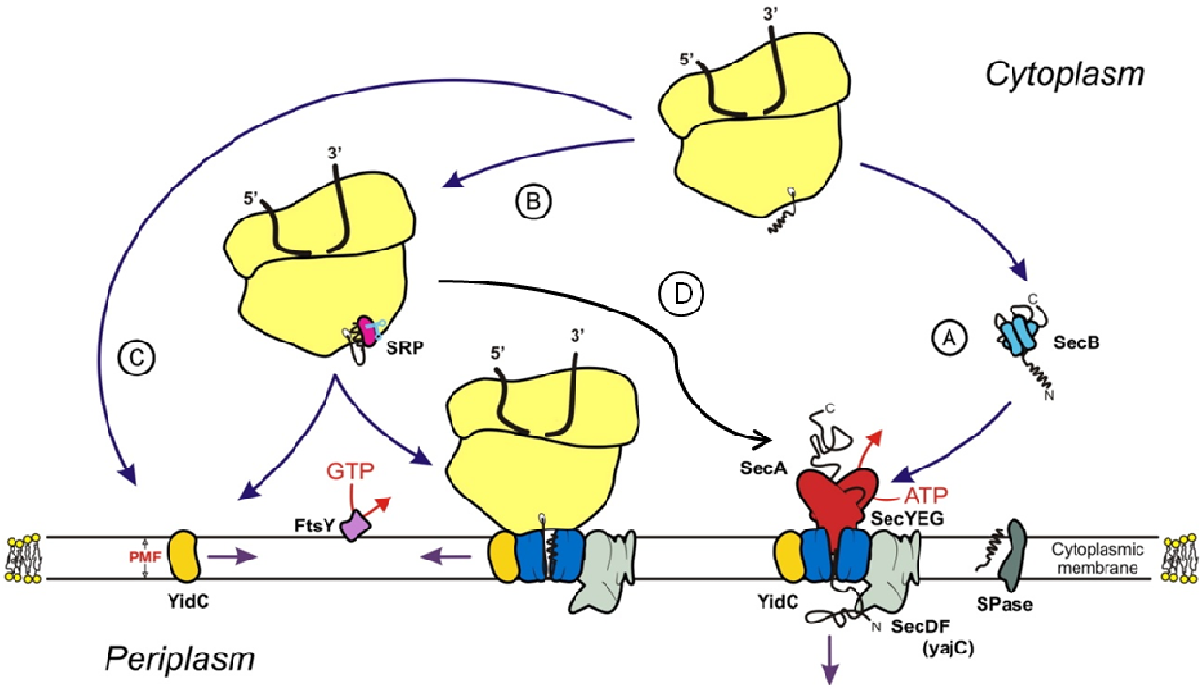

Figure 12. Schematic representation of protein targeting to the Sec-translocase: The bacterial Sec translocon (blue; SecYEG) spans the cytoplasmic membrane (CM). SecA (red; ATPase) acts as secretion motor protein on the cytoplasmic side. Other accessory proteins (yellow: YidC; light grey: SecDFyajC complex) in close proximity to the translocase facilitate the membrane secretion of pre-proteins. Signal sequences of pre-proteins are cleaved at the periplasmic site of the membrane by signal peptidase (SPase). (**A**) Posttranslational targeting of newly synthesised proteins by the chaperone SecB (light blue) to the Sec machinery. (**B**) Cotranslational targeting of the ribosome (light yellow) -nascent peptide chain to the translocase complex via recognition of signal anchor sequence of membrane proteins by SRP (pink) and the SRP receptor FtsY (purple). SecA is again required for the insertion process. (**C**) A subset of membrane proteins can insert into the cytoplasmic membrane via YidC after targeting of the ribosome-nascent chain to YidC. Figure according to [du Plessis et al., 2011]. (**D**) Proposed *M. suis* model for co-translational targeting to the Sec-translocase via SRP-FtsY receptor-binding and SecA interplay.

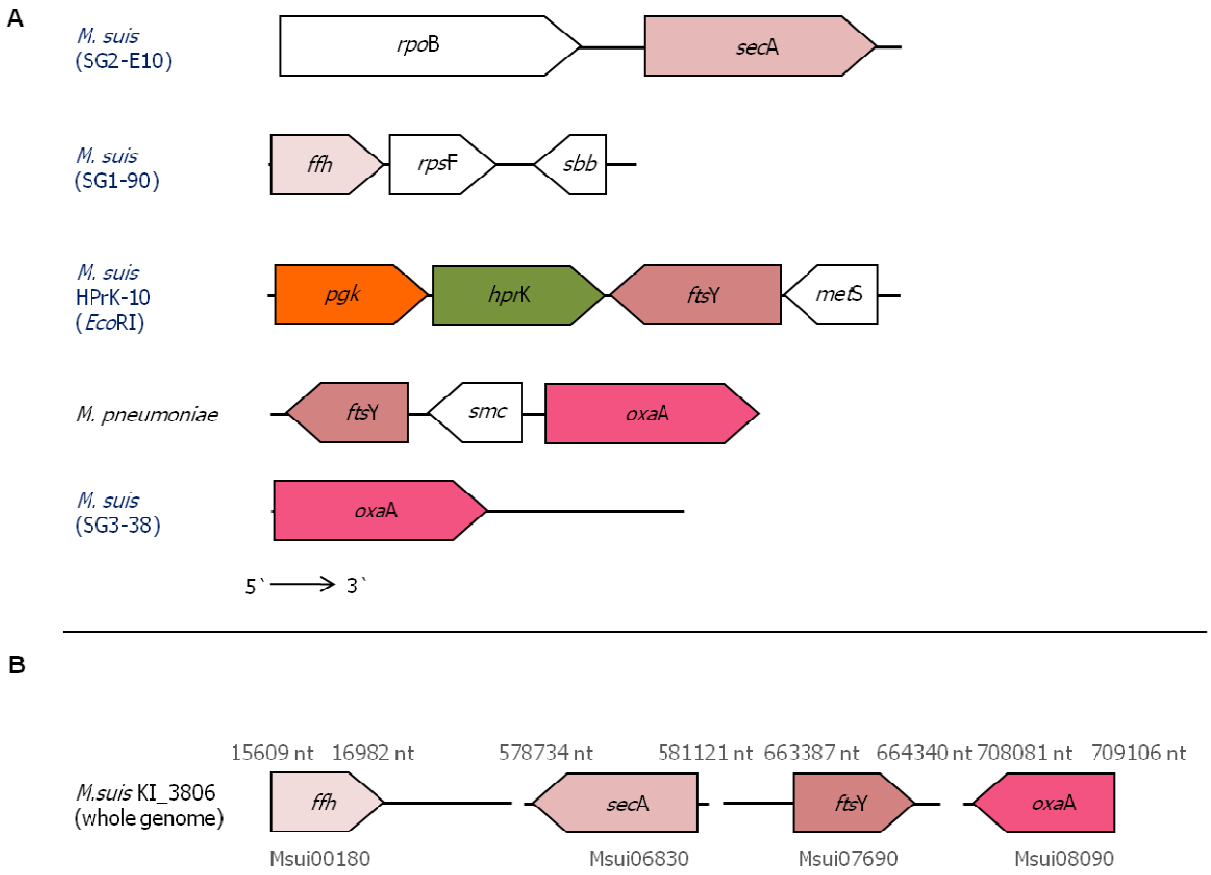

Figure 13. Sec-mechanism: (**A**) Gene organisation of some Sec-associated proteins found in *M. suis* shotgun clones (SG2-E10, SG1-90, SG3-38) and after rehybridisation on genomic *Eco*RI-restricted *M. suis* DNA (HPrK-10). Identified Sec-translocase genes are marked in rosy-pink: *sec*A, ATPase; *ffh*, signal recognition particle SRP; *fts*Y, SRP-receptor protein; *oxa*A, accessory membrane protein YidC. Glycolysis: Pgk (in orange); PTS: HPrkinase/phosphatase (in green). Others: *rpo*B, DNA-directed RNA-polymerase subunit beta; *rps*F, 30S ribosomal protein S6; *ssb*, single-stranded binding protein; *met*S, methionyl-tRNA-synthetase; and *smc*, chromosome segregation protein SMC. (**B**) The same gene organisation was supported by annotation of whole *M. suis* genome (GenBank: FQ790233). All Sec-associated genes are separately found in the *M. suis* genome. The locus_tags and actual nucleotide (nt) positions were placed to the *M. suis* Sec-genes. Only in the genome of *M. pneumoniae* (GenBank: U00089) *oxa*A and *fts*Y were found in close proximity.

6.2.2.4 Phosphate-specific ABC-transporter

The phosphate-specific ABC-transporter (Pst) is a high-affinity ABC-transporter for periplasmic inorganic phosphate (P_i). Usually, Pst-transporters are composed of four proteins (PstS, PstC, PstA, and PstB) which are encoded together with a protein called PhoU in a polycistronic *pst*SCAB-phoU operon (Figure 14) [Lamarche et al., 2008; Ferreira and Spira, 2008]. PstS is a periplasmic protein that binds P_i with high affinity. PstC and PstA form a transmembrane channel through which the P_i molecule is transported by ATPase activity of cytoplasmic PstB. Pst belongs to the phosphate (Pho) regulon and is controlled by the PhoR/PhoB two-component regulatory system (TCRS) shown in Figure 15 [Lamarche et al., 2008]. PhoR is a sensor histidine-kinase for environmental P_i levels. When the P_i concentration in the medium falls below 4 mM, PhoR auto-phosphorylates and activates the response regulator PhoB by phosphorylation leading to up- or down-regulation of target genes of the Pho regulon (by binding of PHO boxes in the promoter region of PHO genes). The function of PhoU is still unclear, but it does not seem to be involved in phosphate transport [Ferreira and Spira, 2008]. When phosphate is in excess, the Pst-transporter and PhoU are thought to form a repression complex with PhoR and are required for dephosphorylation of activated PhoB [Lamarche et al., 2008].

In this study the *M. suis pst*A- *and pst*B-gene were detected by shotgun sequencing (SG1-H1) and Southern Blot hybridisation (SC-195 and PstB-32). As shown in Figure 16, the *pst*B is located behind the *pst*A. Afterwards a putative amino acid permease (*pot*E), the ribosomal protein L17 (*rpl*Q) and HPr (*pts*H) as a part of the PTS system were found in the *M. suis* genome. In comparison to the genome organisation of other related mycoplasmas (i.e. *M. genitalium*, *M. pneumoniae*, and *M. penetrans*) the gene encoding the putative negative regulator protein PhoU was not located in close connection to the other genes of the Pst-transporter (*pts*A and *pts*B). A PhoU homologous gene sequence was identified by whole genome annotation. However, in *M. suis* the PhoU is not located within the *pst*-operon. Furthermore, the PhoR/PhoB two-component regulatory system as shown in Figure 15 seems to be absent in *M. suis* as well as in all other related mycoplasmas.

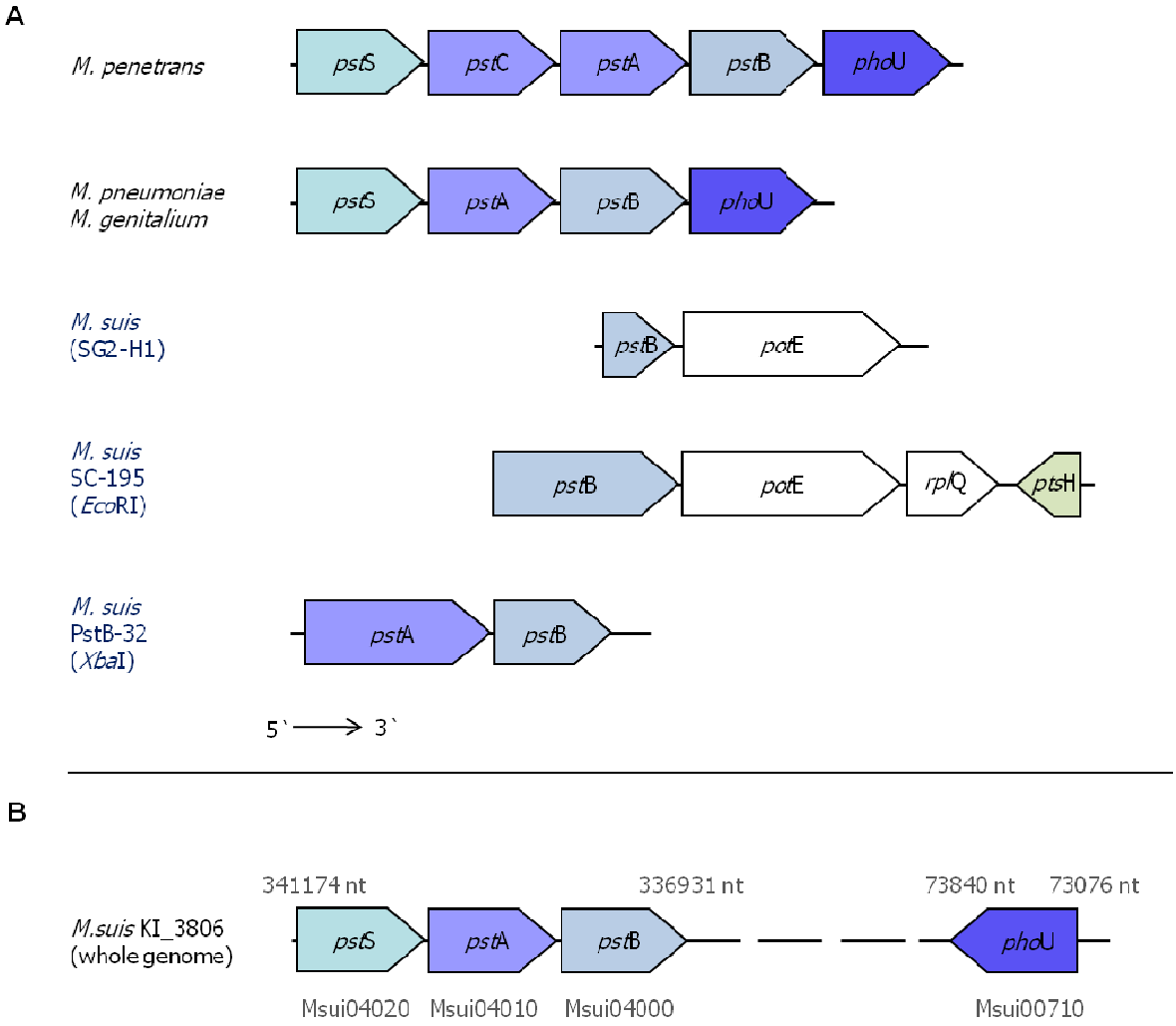

Figure 14. Pst operon: (**A**) Gene organisation of some *pst*-genes found in the *M. suis* shotgun library (SG2-H1) and after rehybridisation on genomic enzyme-restricted *M. suis* DNA (*Eco*RI: SC-195, *Xba*I: PstB-32). Pst genes: *pst*S, substrate-binding protein; *pst*C/A, membrane channel proteins; *pst*B, ATPase. PTS-system: *pts*H, phosphocarrier HPr. Others: *pot*E, putative amino acid permease; *rpl*Q, 50S ribosomal protein L17. In most bacteria and other mycoplasma species (*M. genitalium* (GenBank: L43967), *M. pneumoniae* (GenBank: U00089), and *M. penetrans* (GenBank: BA000026)), the *pst*-genes are organised in a polycistronic *pst*SCAB-*pho*U operon. (**B**) In *M. suis*, PhoU, the putative negative regulator of the Pho regulon, is not encoded together with the subunits of the Pst-transporter as confirmed by whole genome annotation. The locus_tags and actual nucleotide (nt) positions were placed to the *M. suis pst*-genes.

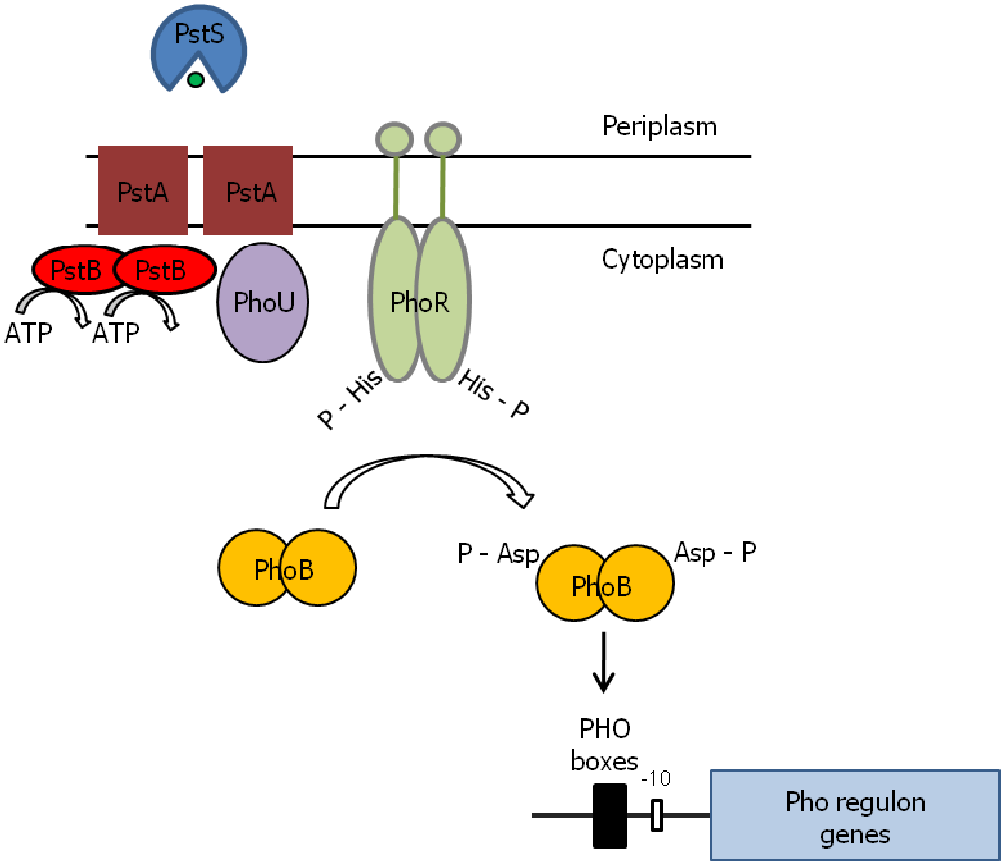

Figure 15. Pho regulon: The high-affinity phosphate (P_i)-specific ABC-transporter Pst interplays with the two-component regulatory system PhoR/PhoB. [Figure according to Lamarche et al., 2008].

6.3 Discussion

In this work shotgun libraries were sequenced and analysed with regard to the *M. suis* metabolism and transport capacities. At the beginning of the work four years ago, whole genome sequences for *M. suis* and, moreover, the technical requirements for the whole genome sequencing of uncultivable bacteria were not available. Therefore, shotgun libraries were constructed and used to gain insight into the biology of *M. suis*. Thereby, several *M. suis* proteins (i.e. HspA1, MSG1, and the inorganic pyrophosphatase) were identified as a prerequisite for their functional characterisation [Hoelzle et al., 2007a+b; Hoelzle et al., 2010].

Sequencing of 600 clones of the shotgun library revealed 78 clones which contained bacterial DNA with the highest homologies to other mycoplasmas and which were therefore assumed as *M. suis* specific clones. The portion of clones (n=522; 87%) which contained eukaryotic DNA was as high as expected from previous studies since the *M. suis* DNA was purified from the blood of experimentally infected pigs [Hoelzle et al., 2007b].

The *M. suis* clones found could be assigned to 72 different proteins e.g. enzymes of the metabolism (e.g. glycolysis), transport systems, proteins of the transcription and translation complex, or ribosomal proteins and DNA-binding proteins. Since the metabolism and the transport capacities of the rather unexplored haemotrophic mycoplasmas are of special interest, clones containing genes encoding for glycolytic enzymes and for transport proteins were chosen for further analyses.

Former *in vivo* and *in vitro* studies indicated that glycolysis is the main ATP generating pathway of *M. suis* [Nonaka et al.; 1996; Smith et al., 1990; Zachary and Smith, 1985]. The present study provided evidence that *M. suis* is a glucose consuming *Mycoplasma* since no other metabolic pathways than glycolysis were found. Furthermore, first evidence for the presence of a glucose-specific uptake system, i. e. the phosphoenolpyruvate (PEP):sugar phosphotransferase (PTS) system in *M. suis* was shown. The two soluble and sugar-unspecific PTS components i.e. enzyme I and HPr as well as the bifuntional HPr-kinase/phosphatase were found in different *M. suis* shotgun library clones. No clones containing other *M. suis* PTS-systems e.g. specific for fructose, mannose or galactose were identified. Comparison of the results of the shotgun sequencing to the meanwhile available whole genome sequences, confirmed these presumptions [Oehlerking et al., 2011]. Therefore, it is most obvious that *M. suis* competes for and scavenges glucose from

the erythrocytic microenvironment. In *B. subtilis* and other Firmicutes as well as in *M. pneumoniae*, genes encoding PTS uptake proteins and glycolytic enzymes are clustered in several operons [Dutow et al., 2010; Ludwig et al., 2001]. In *M. suis*, three gene clusters with glycolytic genes are present: the *tpi*A-*pgm* and the *msg*1-*pgk* clustering is the same like in *M. pneumoniae*, in *M. genitalium* as well as in *M. penetrans*. The latter is common for most bacteria probably reflecting their direct physical interaction in the glycolytic cascade [Dutow et al., 2010; Dandekar et al., 1998]. It is noticeable that the *msg*1-*pgk* operon in *M. pneumoniae* and in *M. genitalium* is directly followed by the *pts*I gene coding for enzyme I of the PTS, and the same cluster in *M. penetrans* by the coding sequence for enolase [Dutow et al., 2010]. The third glycolytic gene cluster of *M. suis* is composed of the *eno*, *pfk*A, and *pyk*F genes. In contrast to this, a third glycolytic cluster was found in *M. pneumoniae* and in *M. genitalium* only for the *pfk*A and *pyk*F genes rather enolase is encoded downstream of the phosphate-specific Pst-transporter.

Beside the glucose-specific uptake system, the shotgun sequencing approach provided information about phosphate transporter, potassium and magnesium transporter, a putative amino acid permease, and a Sec pre-protein translocase. The comparison of these results to the *M. suis* genome sequence showed that the transport capacities of *M. suis* are, in fact, rather restricted to few transport systems i.e. transporters for spermidine/putrescine, haemin, ferrichrome, cobalt, magnesium and probably amino acids and phosphate [Oehlerking et al., 2011].

The general Sec-translocase machinery is responsible for the export of most secretory proteins that are destined for the periplasm or the outer membrane. Sec catalyses the secretion of extracellular toxins, adhesins, pili and other virulence factors and is essential for cell viability [Economou, 2001; Stathopoulos et al., 2000]. Inhibition of the export of such factors could prevent infection implying that the Sec is directly or indirectly involved in bacterial virulence [Stathopoulos, 2000; Stephens and Shapiro, 1997]. The shotgun approach and the subsequent Southern blot analyses revealed the SRP, FtsY, YidC and SecA components of the Sec-translocase machinery. The remaining components SecY and SecE were found in the *M. suis* genome [Oehlerking et al., 2011]. Altogether, no clustering for Sec-genes was observed in *M. suis*. The Sec-associated genes are scattered over the whole *M. suis* genome. *M. suis* might only use the SRP, but not the SecB pathway to target pre-proteins to the Sec-translocase. Beside the soluble cytoplasmic Sec components

(SRP, its receptor FtsY, and the ATPase SecA) a second auxiliary membrane protein translocase YidC was detected in the *M. suis* shotgun library. The channel-forming membrane components, SecY and YecE but no SecG or SecDFyajC-complex were determined by genome annotation. These facts indicate that the Sec-translocase of *M. suis* translocate mainly integral membrane proteins via co-translational targeting (SRP-FtsY receptor binding) into the cell membrane [Muller et al., 2001].

The identified phosphate transporter (Pst) provides *M. suis* with orthophosphate (P_i) which is an essential nutrient for several cell functions and life. Pst belongs to the Pho regulon that monitors primarily phosphate homeostasis. Yet, it is speculated that it might be part of a complex network important for both bacterial virulence and stress response for survival [Lamarche et al., 2008]. Under phosphate-limiting conditions, many bacteria modify their cell surface components. In *B. subtilis*, for example, phosphate-rich teichoneic acids in the cell wall are replaced with phosphate-free teichuronic acids [Pragai et al., 2004]. Moreover, in *S. meliloti*, PhoB positively regulates the synthesis of some exopolysacharides, which are involved in the bacterial invasion of root nodules [Ruberg et al., 1999]. Several studies have described a positive correlation between some *pho* genes and the virulence of some bacteria. Pst mutants showed a reduced resistance to different host defense mechanisms that target bacterial cell surface components [Daigle et al., 1995; Lamarche et al., 2005]. In most bacteria, as well as in other mycoplasmas, Pst-transporters are usually encoded in a polycistronic *pst*SCAB-phoU operon. In the present study the genes of two *M. suis* Pst components PstA and PstB were identified. Southern blot hybridisation analyses further indicated a *pst*-operon in *M. suis.* However, no phoU, the negative regulator of the Pho regulon, was identified within the *M. suis pst*-operon. This finding was confirmed by the genome annotation which demonstrated that the phoU is located in large distance to the *pst*-operon in the *M. suis* genome. Interestingly, the PhoR/PhoB-TCRS was not detected in any *Mycoplasma* species. The displaced *pho*U-gene and the missing *pho*R- and *pho*B-genes raise a lot of questions. It is likely that in mycoplasmas, the Pst-transporter is operating on its own, independently of PhoR/PhoB. Or other common “household” proteins maybe take the part of PhoR/PhoB in order to regulate the Pst-transporter and the expression of Pho-genes.

“Classical” virulence factors such as toxins, haemolysins or haemagglutinins for direct haemolysis are not present in *M. suis*. This leads to the suggestion that some

genes of common metabolic pathways or general transporters, respectively, may exhibit a multifunctional role in inducing/enhancing pathogenic processes during *M. suis* infections. MSG1 and HspA-1 are the best examples for common multitasking proteins in *M. suis*. Both proteins are surface-displayed and probably mediate adhesion to the host cell [Hoelzle et al., 2007a+b]. At the moment, the knowledge about the *M. suis* genome does not allow rendering a statement about potential pathogenicity factors. Thus, the nutrition competition for glucose, amino acids, fatty acids, ribose and nucleotides between *M. suis* and erythrocytes may implicit a new kind of pathogenicity, leading to increasing oxidative stress, shortened life-span and premature removal of erythrocytes, and consequently to anaemia and severe hypoglycaemia.

In summary, the porcine erythrocyte is an extraordinary niche for haemotrophic bacteria, i. e. *M. suis*. More information has to be gained about the minimal set of genes and metabolic processes which are actually necessary for *M. suis* replication and survival in this special environment.

6.4 Conclusions

The results of shotgun sequencing presented here will be completed step by step by analysis of the annotated genome data. Successive reconstruction of the present metabolic pathways in *M. suis* will be helpful in view of identifying the indispensable *M. suis in vitro* growth requirements and investigating the physiology and the virulence-determining factors of *M. suis*.

7. Alpha-Enolase as Virulence Factor in *M. suis*

Various different studies on streptococci, lactobacilli and related *Mycoplasma* agents led us to suppose that *M. suis* α-enolase might have additional functions in pathogenic processes, instead of being just a glycolytic protein in the cytosol. Cross-species Southern blot analysis of *M. suis* genome library clones using an *α-enolase*-specific probe of *M. penetrans,* led to the identification of the library clone SG1-66 that contained a fragmentary open reading frame (ORF) for the *M. suis* α-enolase. We determined the complete *M. suis α-enolase* gene of 1623 bp by rehybridisation on genomic *M. suis* DNA. *M. suis* α-enolase shows immunogenic properties. Transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) elicited that α-enolase is displayed on the *M. suis* surface and might interact with host proteins. For more details refer to the following **paper manuscript 7.1**.

7.1 Paper Manuscript

Surface-displayed α-Enolase of *Mycoplasma suis* exhibits Adhesive Properties

Sabrina A. Schreiner, Albina Sokoli, Kathrin M. Felder, Max M. Wittenbrink, Sarah Schwarzenbach, Katharina Hoelzle, Ludwig E. Hoelzle

Manuscript submitted

Several bacterial glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase, enolase and phospho-fructokinase, are cytoplasmic proteins often presented on the cell surface - despite the lack of typical signal sequences for membrane insertion. There they are suggested to contribute to adhesion to the host cell by interacting with host proteins such as plasminogen, extracellular matrix proteins (i.e. fibronectin), and cytoskeletal proteins (i.e. myosin or actin) as well.
Here we describe the identification and characterisation of α-enolase as a potential *M. suis* adhesin. TEM and CLSM confirmed that α-enolase is surface-localised in *M. suis.* Its surface accessibility was also demonstrated in *E. coli* transformants. Furthermore, *E. coli* transformants expressing a α-enolase-GFP fusion protein were used to study the putative adhesive properties of *M. suis* α-enolase.

Own contributions:
I performed all experiments with the exception of microscopy and FACS analysis, and wrote the manuscript.

Surface-displayed α-Enolase of *Mycoplasma suis* exhibits Adhesive Properties

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ABSTRACT

Mycoplasma suis belongs to the group of haemotrophic mycoplasmas which colonise red blood cells of a wide range of vertebrates. Adhesion to red blood cells is the crucial step in the unique lifecycle of *M. suis*. Due to the lack of a cultivation system, identification of adhesion structures has been difficult. So far, only one adhesion protein i.e. MSG1 was identified. In order to determine further adhesion molecules of *M. suis*, we screened genomic *M. suis* libraries and performed Southern blot hybridisation analyses of genomic *M. suis* DNA. The α-enolase of *M. suis* was identified and analysed genetically and functionally. The encoding gene has 1623 bp in size. The deduced amino acid sequence showed an overall homology of 59.6% to 65.1% to α-enolases of other pathogenic mycoplasmas. The 540 aa *M. suis* α-enolase displays a size extension of about 90 aa in comparison to α-enolases of other mycoplasmas. Recombinant α-enolase expressed *in Escherichia coli* demonstrated immunogenicity in experimentally infected pigs. Immunoblot, confocal laser scanning microscopy and immune electron microscopy analysis using antibodies against recombinant α-enolase, verified the membrane and surface localisation of native α-enolase in *M. suis,* though no typical signal sequences exist. Furthermore, we showed that recombinant α-enolase binds to porcine erythrocyte lysate in a dose-dependent manner. *E. coli* transformants which express α-enolase on their surface, acquire the ability to adhere to porcine red blood cells. In conclusion, our observations indicate that the cell wall-associated form of α-enolase could be involved in the adhesion of *M. suis* to porcine red blood cells.

BACKGROUND

Mycoplasma (M.) suis belongs to the uncultivable haemotrophic mycoplasmas which were primarily found as surface parasites on red blood cells (RBCs) of a wide range of animals (1). Recently a novel *M. suis* isolate has been described which is able to invade RBCs (2). *M. suis* infections manifest as acute febrile haemolytic anaemia in piglets, pregnant sows immediately prepartum, and feeder pigs under stress (1). Clinical signs of chronic low grade infections vary from asymptomatic infections to mild anaemia, mild icterus, growth retardation in feeder pigs, or poor reproductive performance in sows (1). Furthermore, *M. suis* infections are accompanied by a general suppression of the porcine immune system leading to a higher susceptibility to other infections (1, 3, 4, 5). *M. suis* infections are distributed worldwide and cause the pig industry serious economic losses (3, 6). Current studies revealed that *M. suis* also appeared in humans after close contact to *M. suis*-infected animals (7, 8, 9, 10).
The adhesion of *M. suis* is obviously crucial for its unique RBC lifestyle. In electron microscopic investigations, adhesion seemed to be mediated by fine fibrils which bridge a narrow space between *M. suis* and the RBC (1, 11). However, the proteins which could mediate the adhesion as well as the adhesion mechanisms of *M. suis* used, are unknown so far. Research on the *M. suis* pathobiology is still hampered by the lack of an *in vitro* cultivation system. To date, only one *M. suis* protein, i.e. MSG1, has been identified which is able to bind to RBCs. MSG1 is a 36 kDa immunoreactive and surface-localised multitasking protein with enzymatic function in the carbohydrate metabolism (12). For other mycoplasmas, e.g. *M. pneumoniae* and *M. genitalium,* it is known that mycoplasmal cell adhesion obviously is a complex and multifactorial process which requires multiple proteins (i.e. cytadhesins and cytadhesin-associated proteins) (13, 14). Therefore, it is assumed that *M. suis* possesses further adhesion proteins or adhesion-associated proteins. Recently, using proteome and immunome analyses, other immunoreactive proteins of *M. suis* have been identified i.e. GroEL, a protein of the pyruvate dehydrogenase complex, heat shock proteins, and α-enolase (15). The glycolytic α-enolase could be involved in the interaction of *M. suis* with its host cell, since α-enolase of streptococci and *Aeromonas hydrophila* is known to perform an alternative task as a surface protein which binds to plasminogen or fibronectin and thus mediates cell adhesion and invasion (16, 17, 18, 19). Furthermore, the α-enolase of *M. fermetans* is surface-localised and binds to plasminogen, indicating an important role in cell adherence and invasion (20, 21). In

the present study, we have identified the *α-enolase* gene of *M. suis* and recombinant expressed the corresponding protein in *Escherichia coli* for functional characterisation. We described the *M. suis* α-enolase as an immunogenic and surface-localised protein that could act as an adhesion factor in *M. suis*.

MATERIAL AND METHODS

Plasmids, experimental pig sera and *M. suis* isolates

Escherichia (E.) coli K12 strains Top10 and LMG194 (Invitrogen, Basel, Switzerland) were used to clone and express the library clones and the *M. suis α-enolase* gene. For DNA manipulation and protein expression, the following vectors were used: pUC19 (Roche-Diagnostics, Rotkreuz, Switzerland), pGFP (green fluorescent protein; Clontech, Allschwil, Switzerland), and the arabinose-inducible pBadMycHisA (Invitrogen). Blood sera from experimentally infected pigs and *M. suis* isolates were available from previous studies (6, 22).

Purification of *M. suis*

M. suis was purified from the blood of experimentally infected animals. Blood was drawn at maximum bacteraemia as confirmed by microscopic examination of acridine orange-stained blood smears, and by *M. suis*-specific quantitative real-time Light Cycler-PCR (23). Whole blood was collected in Alsever's solution (Sigma, Buchs, Switzerland) as anticoagulant. Purification was performed as described previously (24).

Library construction and sequence analysis

Genomic *M. suis* DNA was purified as described elsewhere (25). Library construction was performed by MWG Biotech (Ebersberg, Germany). Briefly, *M. suis* DNA fragments averaging about 1.5 kb (Ms_library1) and 2.5 to 3.0 kb (Ms_library2) were ligated into a blunt-end cut, *Sma*I-digested pUC19 vector and transformed into *E. coli* Top10.

Identification of *M. suis* α-enolase

For the identification of the *M. suis* α-enolase the *M. penetrans α-enolase* gene (GenBank: BA000026) was amplified and labelled with digoxigenin (DIG)-dUTP (DNA labelling and detection Kit, Roche). For colony hybridisation, the library clones were plated onto LB agar and incubated overnight at 37°C. Colonies were transferred to a nylon membrane (Roche), and lysed following standard methods. After washing, cross linking, and prehybridisation in DIG-Easy-Hyb buffer (Roche) for about 30 min at room temperature, hybridisation of membranes with *M. penetrans* α-enolase probes was performed overnight at room temperature. The library clone SG1-66

reacting with the *M. penetrans α-enolase* probe was sequenced. The containing DNA insert with the highest homology to the *M. penetrans* α-enolase was used to produce a *M. suis*-specific *α-enolase* DIG-labelled probe. Subsequently, *Hind*III-digested genomic *M. suis* DNA was separated on a 0.8% (w/v) agarose gel. After transfer to a nylon membrane by using 1.5 M NaCl (Sigma), 0.25 M NaOH as transfer buffer membranes were incubated with the *M. suis*-specific *α-enolase* probe. The detected 2.1 kb DNA fragment was cloned into pUC19 vector and sequenced. Resulting nucleotide sequences were analysed using the FastA algorithm (Biocomputing, University Zurich, www.bio.unizh.ch). For determination of putative open reading frames (ORFs) we used an ORF finder program (www.ncbi.nlm.nih.gov/projects/gorf/). Translation of ORFs to amino acid sequences was performed by taking into account the alternative genetic codon usage of mollicutes (UGA is read as tryptophan codon (UGA_{Trp}) instead of as a stop codon).

Mutation-combined PCR of the complete *M. suis α-enolase* gene and cloning

To circumvent the *Mycoplasma*-specific translational barrier to the UGA codon (tryptophan *vs.* stop codon) a mutation-combined PCR method (26) was used to convert one TGA triplet within the *M. suis α-enolase* gene into a UGG triplet encoding for tryptophan. The resulting PCR fragments were sequenced and checked for the correct replacement of the UGA triplet by UGG. Using a second PCR round, two restriction sites (*Xho*I and *Pst*I) were added at the 5´ and 3´ ends of the *α-enolase* gene. The restricted and mutated *α-enolase* gene was ligated into pBadMycHisA and transformed into *E. coli* LMG194 (*E. coli_eno*). To construct a α-enolase fusion protein with GFP, we amplified the *gfp* open reading frame from the plasmid pGFP. The amplified *gfp* was ligated into the plasmid pBadMycHisA containing the *α-enolase* gene and into pBadMycHisA without α-enolase (negative control), and transformed into *E. coli* LMG194 (*E. coli_eno_gfp; E. coli_gfp*). The gene sequences and orientations were controlled by sequencing.

Expression and purification of recombinant *α*-enolase of *M. suis* (rMsEno)

Transformed *E. coli* cells (pBad_*eno*, pBad_*eno_gfp*, pBad_*gfp*) were grown to an optical density (OD) $_{600nm}$ of 0.6 in RM medium (Invitrogen) at 37°C. Protein expression was induced by adding 0.02% arabinose (Sigma), and cultures were further incubated for 4 h at 37°C. Protein expression was controlled by 10% (v/v)

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to (27), and by subsequent Coomassie blue staining.

His-tagged proteins were purified by nickel affinity chromatography (Qiagen, Hombrechtikon, Switzerland) from the cytoplasmic (CP) and outer membrane compartments (OM) as described previously (28, 12). Expression of GFP was analysed by fluorescent microscopy using a Leica SP 2 fluorescence microscope (Leica Microsystems, Mannheim, Germany) equipped with filters specific for GFP.

Protein sequencing

Proteins were separated by 10% (v/v) SDS-PAGE. Gels were fixed in 40% (v/v) ethanol (Fluka, Buchs, Switzerland), 10% (v/v) acetic acid, and stained with colloidal coomassie solution overnight. Protein bands were excised from the gel and subjected to in-gel tryptic digestion and extraction of peptides. Peptide mass fingerprint maps were generated by MALDI-TOF mass spectrometry, and MASCOT database search (http://www.matrixscience.com/) was used for the identification of the proteins (TopLab GmbH, Martinsried, Germany).

Production of anti-rMsEno immune serum

A polyclonal mono-specific anti-rMsEno immune serum (RαMsEno) was raised by subcutaneous immunisation of a rabbit with 0.5 mg of purified protein emulsified in a 1:1 ratio with Freund complete adjuvant (Sigma). On days 21, 35 and 49 the rabbit received three additional boost immunisations with rMsEno in Freund incomplete adjuvant (Sigma) in a ratio of 1:1. Ten days after the last boost immunisation the rabbit was bled.

***M. suis* membrane preparation**

To localise α-enolase in native *M. suis* cells, *M. suis* membranes were purified by osmotic lysis (29). Briefly, *M. suis* pellet was suspended in 0.25 M NaCl, and immediately transferred to prewarmed (37°C) deionised water. After shaking (15 min, 37°C) the lysate was centrifuged (40.000 x g, 4°C, 1 h). The cytoplasmic fraction in the supernatant was precipitated by three volumes of acetone (Merck) overnight at 4°C, and the membrane pellet was washed with deionised water and 0.05 M NaCl, 0.01 M sodium phosphate (Sigma, pH 7.5), alternately. Cytoplasmic and membrane

proteins were both resuspended in 500 µl of Beta buffer (0.15 M NaCl, 0.01 M ß-mercaptoethanol (Sigma), 0.05 M Tris-HCl (pH 7.4).

RBC membrane purification

Haemoglobin-free RBC membranes were prepared by hypotonic lysis. Blood was collected from healthy pigs using Alsever´s solution as anticoagulant. RBCs were obtained by centrifugation (500 x g, 15 min) and washed three times in PBS; the leukocyte-rich buffy coat was removed after the first centrifugation step. RBCs were lysed in ice cold lysis buffer (Na-phosphate 5mM, pH 7.6: 4.35 mM Na_2HPO_4 (Sigma), 0.65 mM NaH_2PO_4 (Sigma); 1 mM EDTA) in a ratio of 1:1 (v/v), incubated 5 min at RT under gentle agitation, and membranes were sedimented (45.000 x g, 4°C, 15 min). This lysis step was repeated until haemoglobin was completely removed and the pellet was cleared. Finally, the RBC membranes (designated as porcine erythrocyte lysate, pECL) were resuspended in 2 ml PBS and stored at -80°C until used.

SDS-PAGE, immunoblot, and ligand blot

SDS-PAGE and immunoblots were performed according to standard procedures (27, 30). Immunoblots were probed with *E. coli* absorbed sera from experimentally infected piglets and immunised rabbits (dilution 1:100). The purity of the *E. coli* membrane fractions was verified using a mouse monoclonal antibody to the RNA polymerase beta (RpoB) subunit of *E. coli* (Abcam, Cambridge, UK; 1:1000).

For the ligand blots, rMsEno was biotinylated using the Biotin Protein Labelling Kit (Roche). Ligand blots were then probed with biotinylated rMsEno and peroxidase-conjugated streptavidin (Sigma). Signals were detected by chemo luminescence using home-made ECL solution (solution A: 0.1 M Tris-HCl (pH8.6), 125 mg luminol (Sigma); solution B: 11 mg para-hydroxy coumarin acid (Sigma) in 10 ml DMSO (Sigma); solution C: 35% (v/v) H_2O_2; mixed 10:1:0.003).

ELISA assay for α-enolase-binding

An enzyme-linked immunosorbent assay (ELISA) was used to analyse the binding of rMsEno to RBC membranes (pECL). Microtiter plates (Microlon, Greiner, Nürtingen, Germany) were coated with purified pECL proteins in concentrations ranging from 0.5 to 10 µg serially diluted in carbonate-bicarbonate buffer (15.0 mM Na_2CO_3 (Sigma),

34.9 mM $NaHCO_3$ (Sigma), pH 9.6). Remaining binding sites were blocked with 1% (w/v) proteose peptone (Becton Dickinson, Basel, Switzerland) in PBS. After washing with PBS + 0.05% (v/v) Tween (PBST), purified and biotinylated rMsEno (0.25 µg/100 µl) diluted with 0.5% (w/v) proteose peptone in PBS was added, followed by incubation for 1 h at RT. After washing with PBST, the bound material was detected by using RαMsEno IgG (1:200 in dilution buffer) and horseradish peroxidase-labelled streptavidin (1:2000). Reactions were visualised using ABTS (2, 2`-azinobis-3-ethylbenzthiazoline-sulfonic acid; Roche) according to the manufacturer's recommendations, and OD values were measured at 405 nm by a computer-assisted micro plate reader (Tecan, Männedorf, Switzerland). Inhibition of binding was monitored by pre-incubating biotinylated rMsEno or RαMsEno serum with rMsEno or RαMsEno IgG, respectively (1:100 in dilution buffer). Rabbit pre-immune serum served as negative control.

Dot blot analysis

Surface localisation of α-enolase in *E. coli* was checked by dot blot. Arabinose-induced and non-induced *E. coli_eno* were dropped onto a nitrocellulose membrane, and incubated with RαMsEno (pre-adsorbed with *E. coli*). The intactness of the *E. coli* cells was verified by using the anti-RpoB monoclonal antibody of *E. coli* (data not shown).

Adhesion of recombinant *E. coli_eno* to RBCs

Attachment of *E. coli_eno* to porcine RBCs was performed using GFP recombinants (*E. coli_eno_gfp* and *E. coli_gfp*). Thin porcine blood smears were incubated with bacteria for 30 min at 37°C. To eliminate unbound bacteria, blood smears were washed five times with PBS. Adhesion was visualised by using a Leica SP 2 fluorescence microscope, and images were captured by using Image J program and Imaris Software with a Soft Imaging System (SIS) camera, and the analysis FIVE Software (Soft Imaging System GmbH, Münster, Germany).

Immunogold transmission electron microscopy (TEM)

RBCs of *M. suis*-infected blood were diluted (1:10) in PBS and settled on 12 nm carbon-coated cover slips (1200 x g, 5 min) using a Cytospin 2 centrifuge (Shandon, Dako-Diagnostica, Zug, Switzerland). Unreacted aldehydes were blocked with 0.1 M

glycine (Roth) in PBS (10 min). Non-specific binding was reduced by incubation of samples in 3% PBS-buffered foetal calf serum (FCS, Biochrom, Schaffhausen, Switzerland; 40 min). Then, RBCs were stained with RαMsEno (1:100; 1 h), followed by gold-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:100; 1 h; Sigma).
After immunostaining, samples were fixed with 2.5% PBS-buffered glutaraldehyde solution (GA grade I; Sigma), and post-fixed in 1% osmium tetroxide (Fluka Chemie, Buchs, Switzerland). Finally, the cover slips were dehydrated with increasing concentrations (70-100%) of ethanol, embedded in epon/araldite (Fluka), and sectioned. The grids with ultrathin sections were contrasted with 4% uranyl acetate (Fluka Chemie, Buchs, Switzerland) and examined using a Phillips CM 100 transmission electron microscope.

Confocal laser scanning microscopy (CLSM)

RBCs of *M. suis*-infected blood (40 µl) were diluted in 2.5 ml PBS containing 10 mM glucose and 0.1% BSA (Sigma). Cells were fixed in 4% PBS-buffered paraformaldehyde (Sigma) containing 0.01% GA (grade I) and seeded onto poly-L-lysine-coated glass slides (SuperFrost; Menzel, Braunschweig, Germany). Unreacted aldehydes were blocked with 0.1 M glycine (Carl Roth, Karlsruhe, Germany) in PBS (40 min). Non-specific binding of antibodies was reduced by incubation of samples in 3% FCS in PBS (1 h). The RBC surface and *M. suis* cells were stained with purified mouse anti-pig CD235a (glycophorin A) monoclonal antibody (1:100; Phar-Mingen, BD Biosciences, Europe) and RαMsEno serum (1:100; 1 h), followed by tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-mouse IgG (1:100, Sigma), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma), respectively, (1 h). The staining procedure resulted in TRITC-labelled RBCs (red) and FITC-labelled *M. suis* cells with the surface-exposed α-enolase (green), respectively. Confocal microscopy was performed with a Leica SP 2 confocal microscope.

RESULTS

Cross species identification of *M. suis α-enolase*

In order to identify the *M. suis α-enolase* gene, we screened genomic *M. suis* DNA libraries by Southern blot analysis using a DIG-labelled *M. penetrans α-enolase* probe. Sequence analyses of the reacting library clones revealed that the clone SG1-66 contained an insert with highest homology (61.0 %) to the *α-enolase* gene of *M. penetrans*. The clone SG1-66 was completely sequenced. The 1794 bp insert of SG1-66 has an average G+C content of 35.86% and includes two incomplete open reading frames (ORF), i.e. the incomplete ORF of 1080 bp for the *α-enolase* gene and a second incomplete ORF of 607 bp for the *pfk*A (phospho-fructokinase A). The entire *α-enolase* ORF was obtained by Southern blot analysis of genomic *M. suis* DNA incubated with a 920 bp *M. suis α-enolase* probe derived from SG1-66 (Figure 1). The reacting 2.1 kb hybridising band was cloned and sequenced. The resulting clone *eno-Hind*III was found to contain two ORFs for hypothetical proteins and the N-terminal portion of the *M. suis α-enolase*. By reconstruction of the genomic DNA sequences from SG1-66 and *eno-Hind*III, the entire 1623 bp ORF for the *M. suis* α-enolase was found (Figure 1) encoding for a 540 aa protein with a predicted molecular mass of 58.7 kDa and an isoelectric point of 5.80. The predicted α-enolase showed the highest homology with α-enolases of *M. penetrans*, *M. capricolum*, and *M. mycoides* (65.1%, 59.6%, and 58.6%, respectively).

The 540 aa *M. suis* α-enolase displays a size extension of about 90 aa in comparison to α-enolases of other mycoplasmas which range from 450 aa (*M. penetrans*) to 458 aa (*M. genitalium*). The corresponding amino acid sequence alignment and analysis are illustrated in Figure 2. The characteristic and highly conserved α-enolase signature motif (aa-sequence VLIKvNQIGTLSET) was identified in *M. suis* α-enolase by using the program PROSITE of Swiss Institute of Bioinformatics (www.expasy.org/cgi-bin/prosite/ScanView). The substrate-binding pocket, the cofactor-binding site for Mg^{2+} and the dimer interface region was also found in the *M. suis* α-enolase.

Expression and characterisation of recombinant *M. suis* α-enolase in *E. coli*

In order to circumvent the inability to express *Mycoplasma* proteins in *E. coli* due to the atypical UGA codon usage (translation table 4; UGA is translated as tryptophan instead of stop), a mutation-combined PCR was used to replace the one UGA triplet

(aa position 389) by an UGG triplet. The overexpression of the *M. suis* α-enolase (rMsEno) in *E. coli* resulted in a protein migrating on SDS-PAGE with a molecular mass of 63 kDa (Figure 3A).

The recombinant protein was used to raise an *M. suis* α-enolase-specific rabbit polyclonal antiserum (RαMsEno). The specificity of RαMsEno was demonstrated by immunoblot analysis. RαMsEno specifically reacted with the 63 kDa band of rMsEno, and with a 59 kDa band of *M. suis* whole cell preparation (Figure 4A+B). The size shift between the rMsEno and the *M. suis* α-enolase is caused by the 6-His tag and the *myc* epitope fused to rMsEno. The specific immunoreactivity of rMsEno was confirmed by immunoblot using five sera of an experimentally *M. suis*-infected piglet, taken during the time course of the disease, and sera of three non-infected piglets as negative control (Figure 3B). Only the sera of the *M. suis*-infected piglet showed clear reactions with rMsEno.

Localisation of rMsEno in *E. coli*

The sub cellular localisation of α-enolase in *E. coli* transformants was analysed by immunoblot analysis using *E. coli* cytoplasm and membrane preparation, and the RαMsEno. As shown in Figure 4B, a 63 kDa band corresponding to rMsEno could be detected in both purified compartments. The purity of the membrane fraction was demonstrated by immunoblot analysis using a monoclonal antibody against the intracellular RNA polymerase beta subunit (RpoB) of *E. coli*. A reaction was observed with the cytoplasmic fraction, while no reaction was found with the membrane preparation (Figure 4C). Dot blot analysis with intact recombinant *E. coli* cells provided evidence of surface localisation of rMsEno in *E. coli*. Arabinose-induced *E. coli_eno* cells showed specific reactions with RαMsEno (Figure 4D) indicating the surface expression of α-enolase in *E. coli* transformants. In contrast, non-induced *E. coli_eno* did not react with RαMsEno.

Localisation of α-enolase in *M. suis*

To localise α-enolase in *M. suis* whole cell, cytoplasmic, and membrane protein preparations were analysed. The hyper immune serum specifically reacted with the 59 kDa α-enolase in all three preparations, reinforcing the evidence of cytoplasmic and membrane-localised *M. suis* α-enolase (Figure 4A). The surface exposure of *M. suis* α-enolase was demonstrated by TEM analysis of immunogold-stained

M. suis-infected porcine RBCs. Gold-labelled *M. suis* α-enolase was observed on the surface of *M. suis* cells near the attachment site to the RBC (Figure 5). In addition, surface accessibility of *M. suis* α-enolase was further proven by CSLM (Figure 6).

Alpha-enolase is involved in attachment to porcine RBCs

The binding activity of rMsEno to pECL was tested by ELISA. Microtiter plates were coated with decreasing concentrations of purified pECL proteins and incubated with biotinylated rMsEno. Purified rMsEno bound *in vitro* to pECL in a dose-dependent manner (Figure 7A). The rMsEno binding to the lysate was specifically blocked by pre-incubation of the protein with RαMsEno. A clear reduction of binding (43.13% +/- 6.5% standard deviation) was measured compared to rMsEno without RαMsEno serum incubation (Figure 7B).

The rMsEno binding to the porcine RBC membrane was further specified by ligand blot using pECL as antigen and biotinylated rMsEno (Figure 8). Four pECL bands were detected by chemo luminescence specifically reacting with rMsEno. Maldi-TOF mass spectrometry analysis revealed for the four proteins homologies namely to porcine leukocyte elastase inhibitor (59 kDa), ß-actin (45 kDa), GAPDH (34 kDa), and S100 calgranulinB-like protein (20 kDa).

***E. coli* transformants adhere to porcine RBCs**

Adhesion of *E. coli_eno_gfp* was visualised by fluorescence microscopy. Alpha-enolase expressing *E. coli* transformants specifically adhered to porcine RBCs. The control (*E. coli_gfp*) showed no adhesion (Figure 9).

DISCUSSION

Expression of virulence factors and attachment of bacteria to host cells, in the case of *M. suis* to porcine RBCs, are initial and crucial steps for the onset of disease. Until now, the identification of proteins which are involved in the adhesion of haemotrophic mycoplasmas and *M. suis* as well, has been hampered mainly by the lack of *in vitro* cultivation systems and the related drawbacks.

Previously, a serological proteome analysis of *M. suis* purified from an acutely diseased pig and reconvalescent pig sera clearly indicated that *M. suis* α-enolase is an immunoreactive protein (15). For many infectious agents surface-displayed α-enolase has been described to function as adhesin (31, 32, 33). Housekeeping enzymes such as α-enolase, glyceraldehyde-3-phosphate dehydrogenase or phospho-glycerokinase are supposed to carry out various additional and virulence-associated functions beside their common activities, i.e. in glycolysis (31). Therefore, the present study aimed at the identification and characterisation of the *M. suis* α-enolase. Until now, one useful strategy for the investigation of putative *M. suis* adhesins and virulence factors was the screening of genomic *M. suis* DNA shotgun libraries (12, 15). In doing so, we found a library clone (SG1-66) containing an incomplete ORF encoded for the α-enolase protein of *M. suis.* By means of Southern blot hybridisation of genomic *M. suis* DNA the whole *α-enolase* nucleotide sequence of 1623 bp could be determined for *M. suis*. The characteristic enolase signature motif and the three functional sites for cofactor-binding (Mg^{2+}), substrate binding (PEP) and homodimerisation are present in the deduced aa sequence of *M. suis* α-enolase. Like that of *M. fermetans* or *Streptococcus pneumoniae* the *M. suis* α-enolase lacks a signal sequence or the typical motifs required for membrane anchoring (20, 34). Therefore, it is supposed that these proteins form a new group of surface proteins which uses an as-yet-unknown mechanism for secretion and reassociation with the bacterial cell membrane/surface; they are often displayed on the cell surface in order to execute an infection-related function (35, 32, 18). Furthermore, when compared to α-enolases of other related mycoplasmas, *M. suis* α-enolase possesses an extended C-terminus of about 90 aa. The protein in *M. suis* reacting with the RαMsEno showed a molecular mass of 59 kDa which corresponds to the predicted size based on the deduced aa sequence. Therefore, no obvious posttranslational modification occurs. At the moment nothing is known about putative functions of this extension and, therefore, further investigations are necessary. We

could suggest that the C-terminal tail might potentially allow *M. suis* α-enolase to interact with other proteins in the cytoplasm and overall *M. suis* to interfere with proteins of the porcine RBC membrane (due to its surface accessibility on the *M. suis* cell surface).

Our immunological data demonstrated that *M. suis* α-enolase exhibits strong immunogenicity. The recombinant protein in *E. coli* specifically reacted with sera of an *M. suis*-infected piglet, taken during the time course of the disease (Figure 3B). Accordingly, in *M. synoviae* for example, α-enolase has also been identified as one of its major immunogenic proteins (36). To date, two recombinant proteins of *M. suis* i.e. MSG1 and HspA1 are evaluated as diagnostic antigen (12, 15, 37), and a third recombinant antigen in terms of the *M. suis* α-enolase could be an improving complement for the secure specific and sensitive serological diagnosis of *M. suis* infections in future.

Basically, proteins which are acting as potential adhesins have to be localised on the cell surface of pathogenic bacteria. By using immunoblot and immune electron as well as confocal laser scanning microscopic analyses, we determined the α-enolase in the cytoplasm, in the membrane, and on the surface of *M. suis*. Due to the surface expression of *M. suis* α-enolase, we supposed a multifunctional potential in addition to its common glycolytic activity in the cytoplasm. Since verification on the actual role of α-enolase in *M. suis* is still hampered by the lack of *in vitro* culture systems we used *E. coli* transformants as model system. In *E. coli* rMsEno was expressed in the cytoplasm as well as in the membrane (Figure 4B). We further verified the surface accessibility of the rMsEno in the *E. coli* transformants by dot blot analysis. The fact that a cytoplasmic glycolytic protein is expressed on the surface of gram-negative bacteria is out of the ordinary, especially due to the lack of classical signal sequences. However, the same phenomenon was found for the GAPDH-like *M. suis* protein MSG1 indicating a unique expression profile for *M. suis* (12). The membrane localisation and the surface accessibility of α-enolase make evident that this protein has additional non-enzymatic functions in *M. suis*. The participation of α-enolase in the *M. suis* adhesion process to porcine RBCs was indicated by two factors: (i) non-adherent *E. coli* cells acquire the ability to bind to RBCs due to the expression of α-enolase on their surface, and (ii) rMsEno binds to immobilised RBC lysate. This binding was significantly and specifically reduced by anti-α-enolase antibodies as well as by rMsEno. The binding of the *M. suis* α-enolase was further characterised by

ligand blot analysis. Four interacting proteins could be identified as porcine leukocyte elastase inhibitor (LEI), ß-actin, GAPDH, and S100 calcium-binding (calgranulinB-like) protein. LEI and S100 are proteins associated with inflammatory and anti-inflammatory processes. ß-actin is part of the cytoskeleton and GAPDH is a key glycolytic enzyme. Further studies are needed to evaluate whether α-enolase could be part of an adhesion complex which is described for other mycoplasmas (13, 14). Various proteomic studies with regard to the glycolytic pathway revealed that glycolytic enzymes often assembled in complexes in the cytoplasm to ensure higher glycolytic fluxes than non-interacting enzymes (38, 39, 40). Kuhner et al. (41) proposed two glycolytic enzyme complexes in *M. pneumoniae*. One of these complexes is composed of GAPDH, Pgk, α-enolase, and chaperone protein DnaK. The presence of such complexation of *M. pneumoniae* glycolytic proteins was further supported by the findings of Dutow et al. (38). They depicted α-enolase as the core protein that is capable of strongly interacting with all other glycolytic enzymes. It seems likely that similar glycolytic enzyme complexes around α-enolase might be formed in *M. suis* in order to act as complex adhesion apparatus. Since the *M. suis* GAPDH protein MSG1 was also described as a surface-localised adhesion protein, it might be suspected that both MSG1 and α-enolase participate in complexation-mediated adhesion (12).

In summary, we identified the 59 kDa *M. suis* α-enolase as a putative multitasking glycolytic protein showing characteristics of adhesion to porcine RBCs. It is indispensable to identify the actual α-enolase binding partner on the porcine RBC, as well as other parts of a putative adhesion complex. The knowledge of this complex interplay between pathogen and host will allow a better understanding of the pathogenesis of *M. suis*-induced diseases and will help to develop prophylactic reagents i.e. vaccine.

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FIGURES

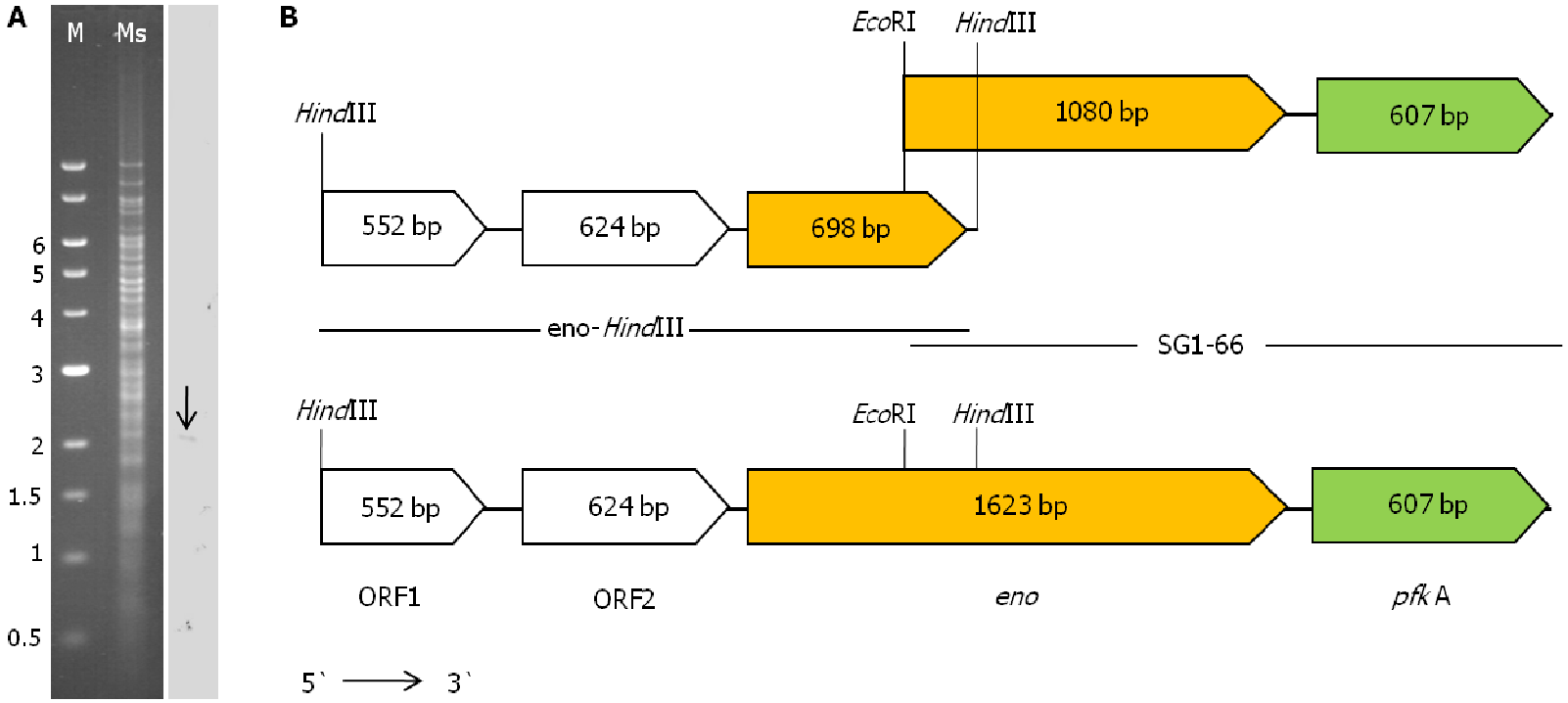

Figure 1 Cross species Southern blot hybridisation and genome organisation of *M. suis* α-enolase. (A) Agarose gel electrophoresis and hybridisation of *Hind*III-restricted genomic *M. suis* DNA with the DIG-labelled *M. suis α-enolase* probe detected a ~2100 bp DNA fragment (arrow); (B) genomic location of *M. suis α-enolase*: Shotgun library clone SG1-66 carries two incomplete *M. suis* ORFs for α-*enolase* (1080 bp) and for *phospho-fructokinase* A (607 bp) in its insert. Clone eno-*Hind*III obtained from rehybridisation contains two ORFs for hypothetical proteins and an incomplete ORF for *M. suis* α-*enolase* (698 bp). Both clones together resulted in the complete *M. suis* α-*enolase* gene of 1623 bp in size. M, molecular weight marker (kDa); Ms, *Hind*III-digested *M. suis* DNA.

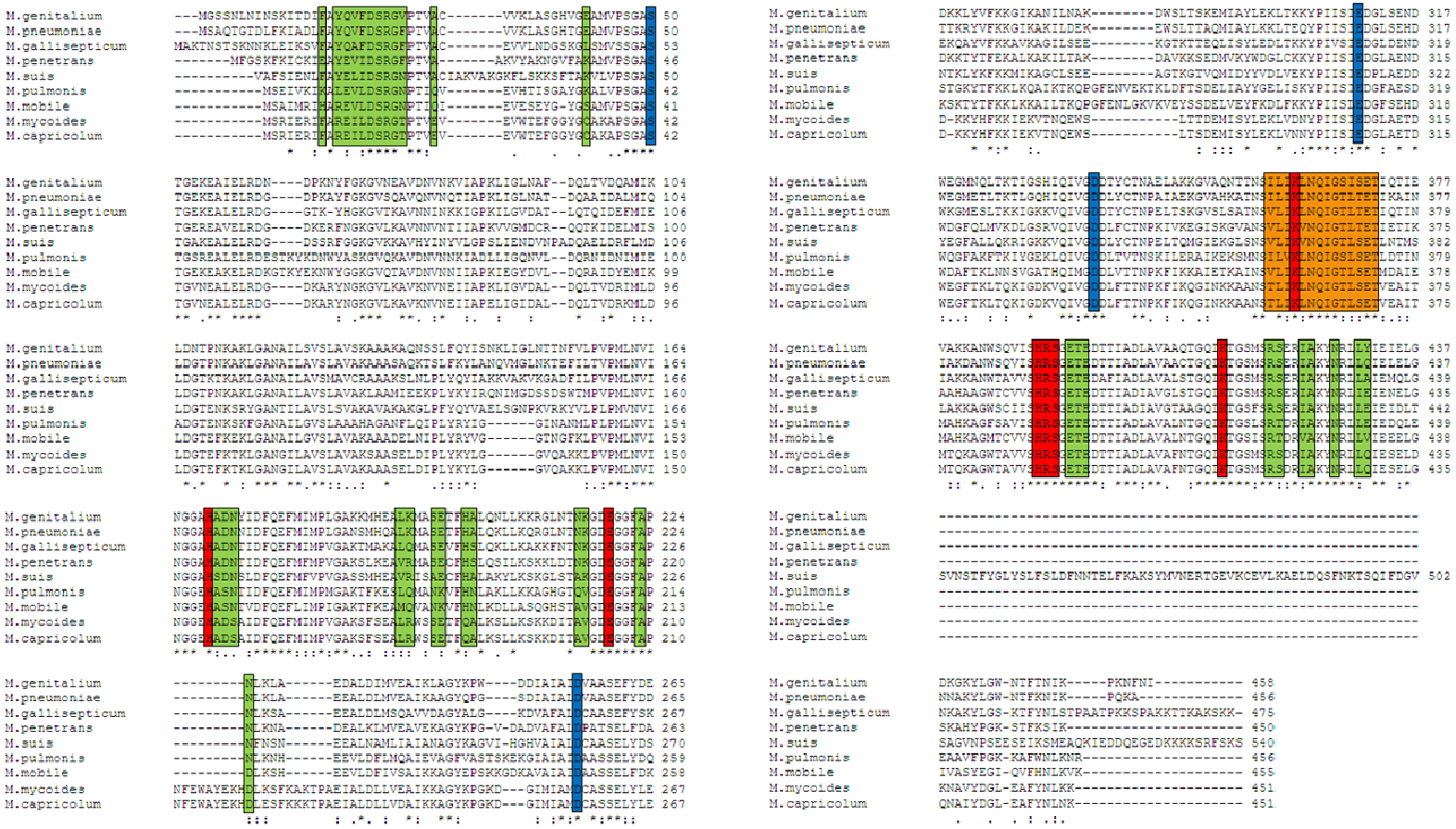

Figure 2 Amino acid sequence aligment (ClustalW) of *M. suis* α-enolase with α-enolases of related mycoplasmas. The highly conserved enolase signature motif (VLIKvNQIGTLSET) and the three functional binding sites are signed by coloured boxes: orange, enolase signature at aa position 364-377; red, substrate-binding pocket at aa positions 171, 221, 367, 395...397, and 418; blue, Mg^{2+}-binding site at aa positions 50, 261, 315, and 342; green, dimer-interface region at aa positions 9, 11...19, 23, 42, 172...174, 195, 196, 199, 200, 203, 204, 217, 218, 225, 227, 399-401, 424...426, 428, 429, 432, 435, and 436; in green. The *M. suis* α-enolase protein has an extended C-terminus of about ~90 aa.

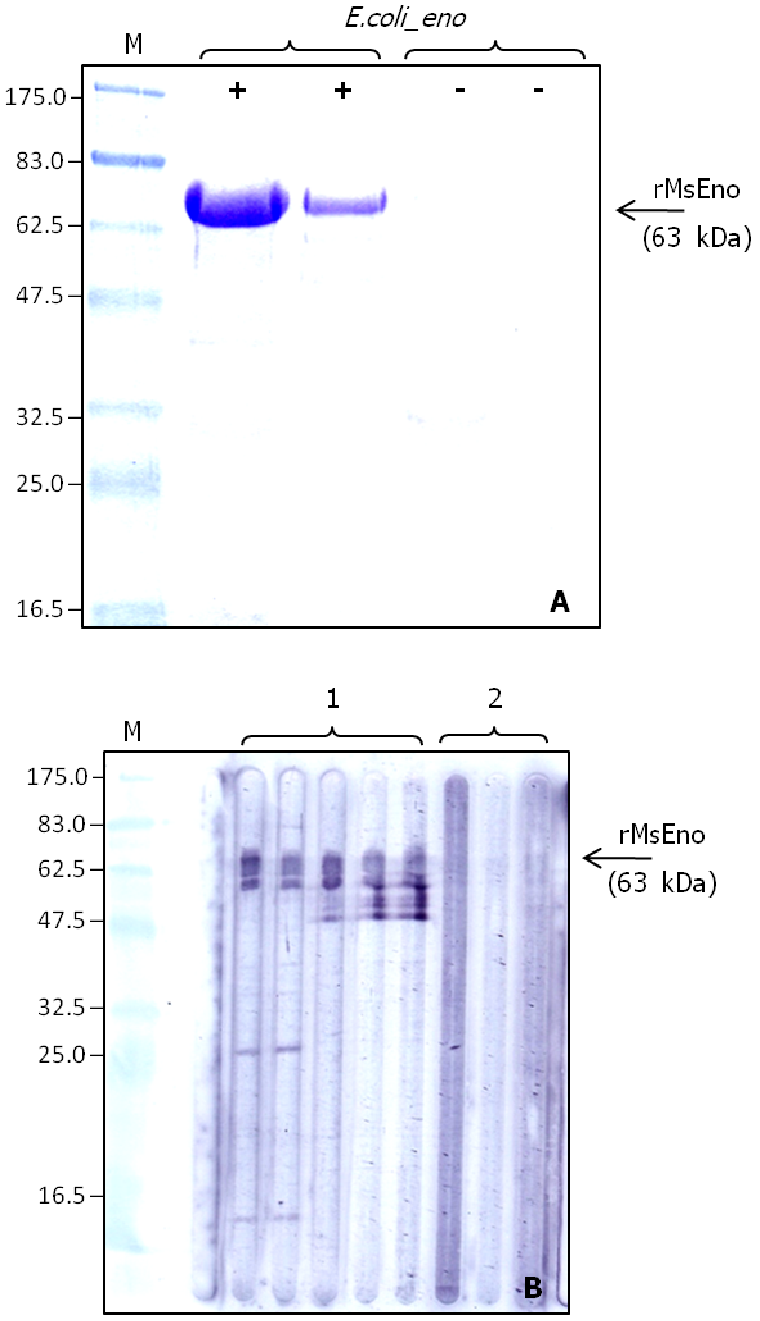

Figure 3 Immunogenicity of *M. suis* α-enolase. (A) SDS-PAGE of recombinant *M. suis* α-enolase (rMsEno); in arabinose-induced (+) and non-induced (-) *E. coli_eno* transformants; (B) Western blot (multiscreen) analysis of rMsEno reacting with (1) five sera of *M. suis*-infected pigs and (2) sera of three *M. suis*-negative pigs as negative controls. M, molecular weight marker (kDa).

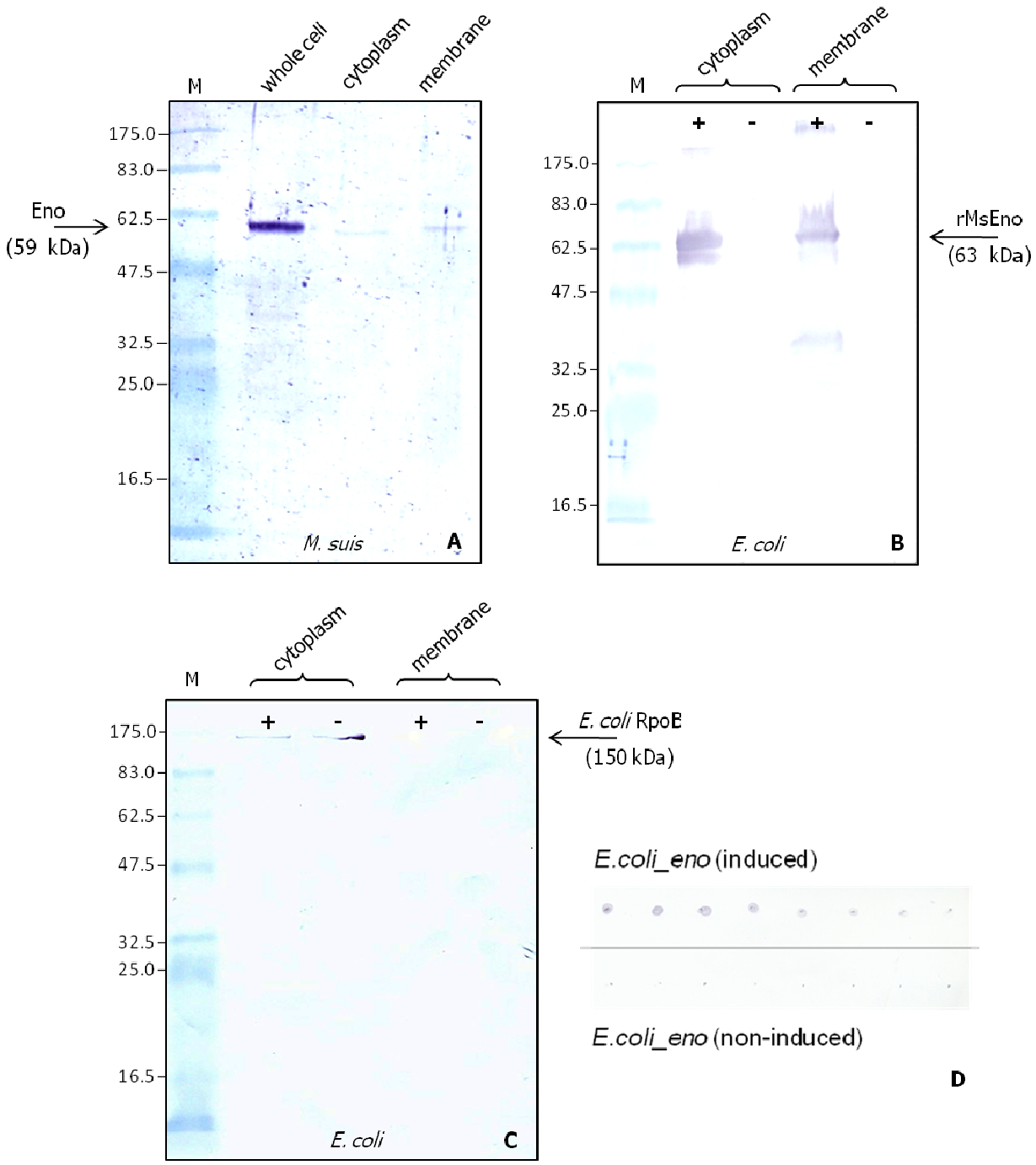

Figure 4 Immunoblot analysis of the sub cellular localisation of α-enolase in *M. suis* and in *E. coli*. (A) *M. suis* α-enolase (Eno) was found in all three *M. suis* fractions (*M. suis* whole cell, cytoplasm, and membrane); (B) Arabinose-induced (+) *E. coli_eno* transformants expressed rMsEno (~63 kDa) in both, cytoplasm and membrane. Non-induced (-) *E. coli* cells showed no rMsEno expression; (C) To control the intactness of the membrane fraction a second *E. coli* immunoblot was performed with a monoclonal antibody against *E. coli* RpoB. Only the cytoplasmic fraction reacted with the antibody; (D) Dot blot analysis: induced and non-induced *E. coli_eno* whole cell transformants were spotted onto a nitrocellulose membrane and reacted with RαMsEno. M, molecular weight marker (kDa).

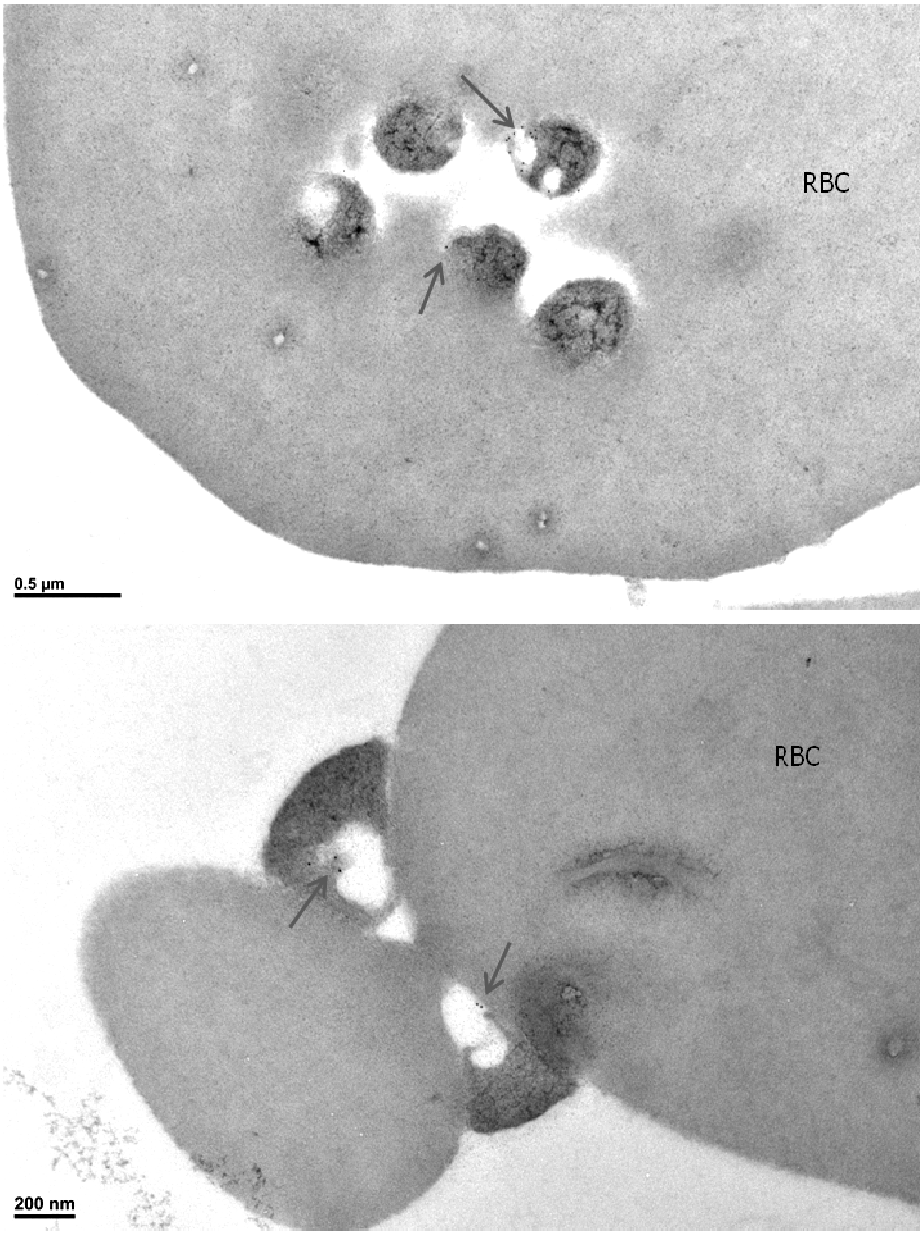

Figure 5 Immunogold transmission electron micrographs of surface-displayed *M. suis* α-enolase. Gold particle bound to *M. suis* α-enolase are accumulated on the attachment site between *M. suis* and porcine RBCs (indicated by arrows).

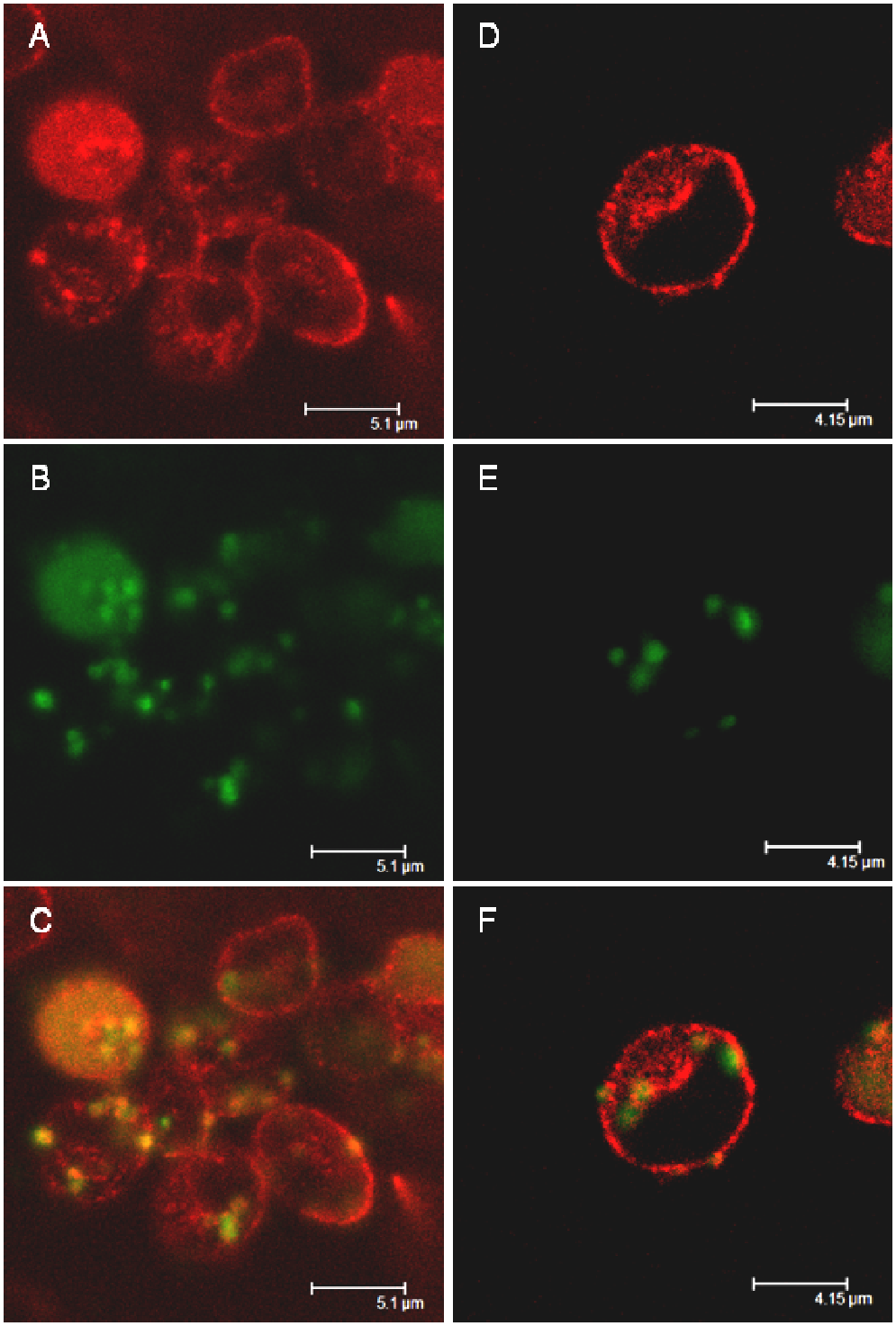

Figure 6 Confocal laser scanning micrographs of surface-displayed *M. suis* α-enolase. (A and D) TRITC-labelled porcine RBCs due to glycophorin A (red fluorescence); (B and E) FITC-stained *M. suis* cells due to surface-accessible α-enolase (green fluorescence); (C and F) superimposition of red and green fluorescence images clearly resulted in distinguishable green fluorescent dots demonstrating surface exposure of *M. suis* α-enolase.

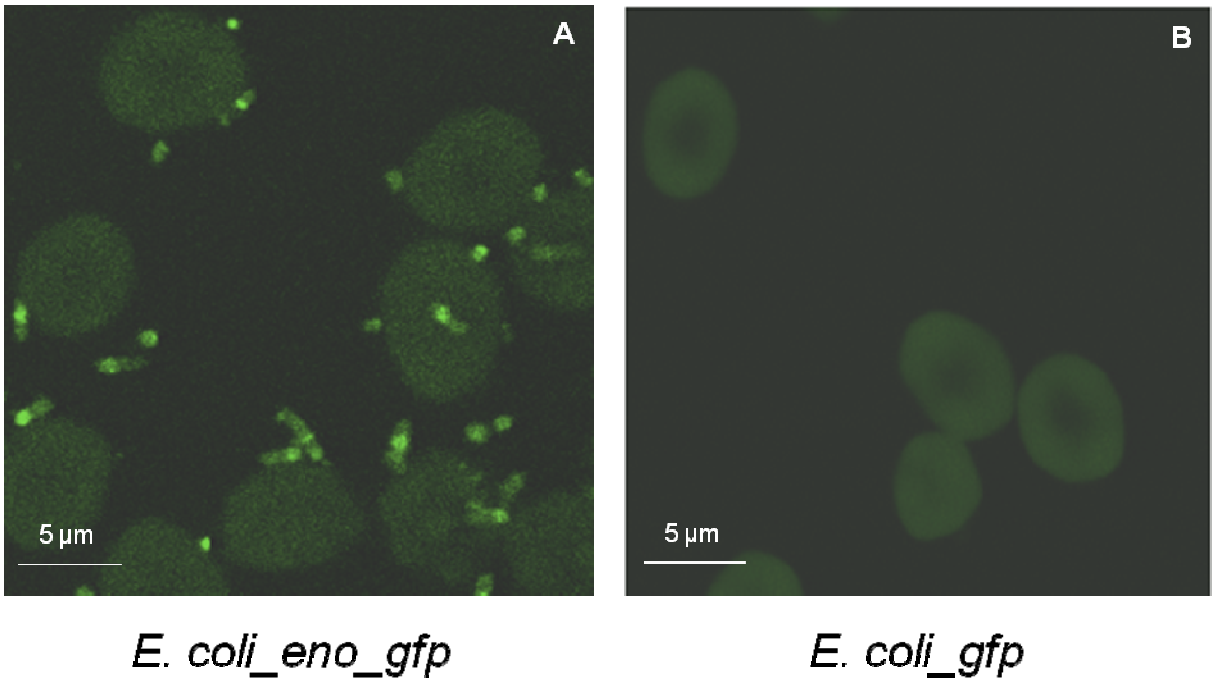

Figure 7 Fluorescence micrographs showing adhesion to pig RBCs. (A) *E. coli* transformants expressing *M. suis* α-enolase and GFP as fusion protein (*E. coli_eno_gfp*) adhere to RBCs; (B) *E. coli* transformants expressing GFP alone (*E. coli_ gfp*) show no adhesion.

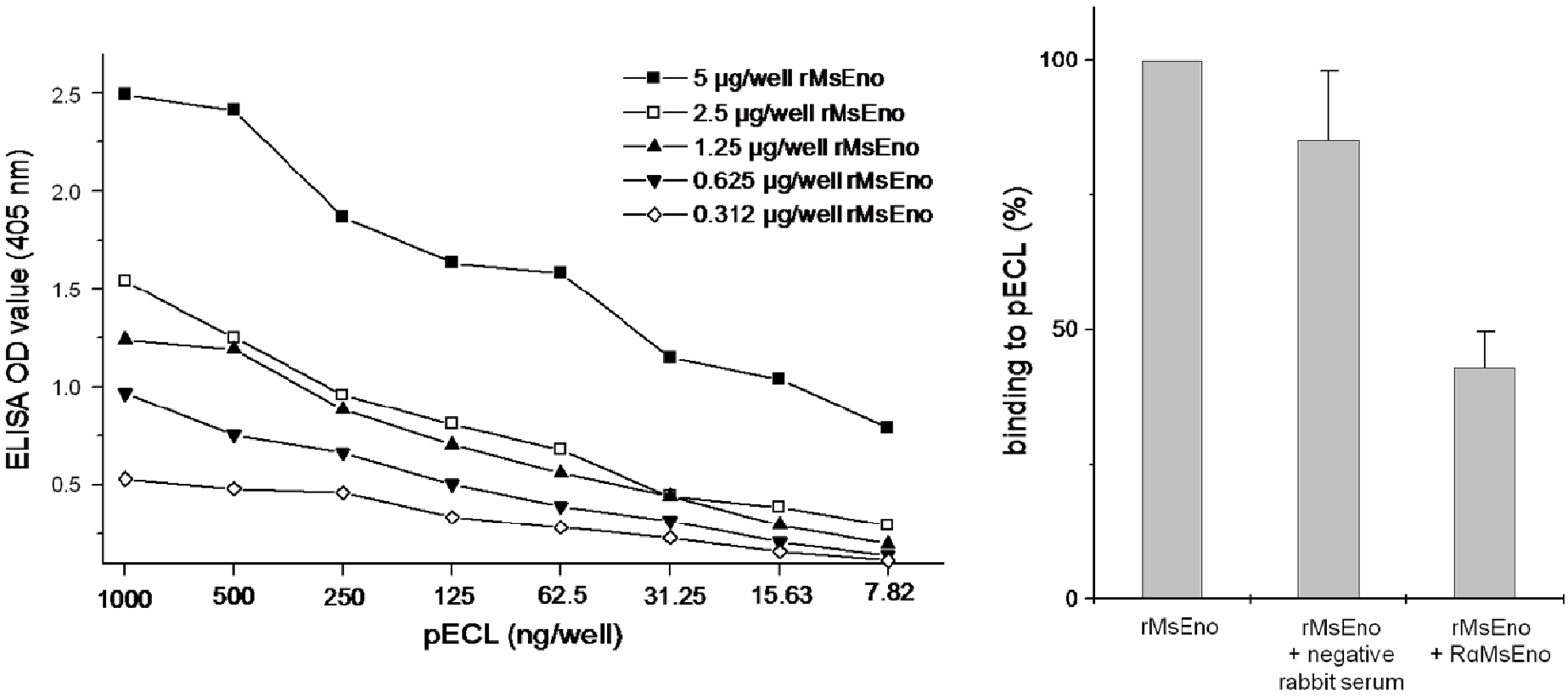

Figure 8 ELISA: Binding of recombinant enolase (rMsEno) to porcine erythrocyte lysate (pECL). (A) Microtiter plates were coated with 1 µg pECL (diluted in log2 dilution steps), and decreasing concentrations of rMsEno were added to each well. Bound protein was visualised using biotinylated RαMsEno and peroxidase-labelled streptavidin. Values represent the average of three independent assays. (B) Inhibition of rMsEno binding to pECL by pre-incubation with RαMsEno. Each bar represents the mean (plus standard deviation) binding values from three independent experiments in relation to 100% binding capacity to pECL without any serum pre-incubation.

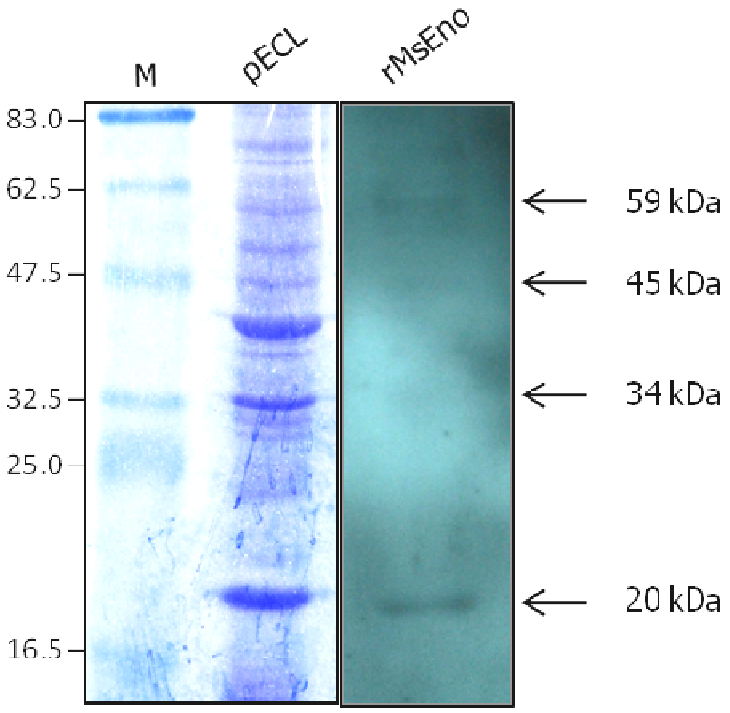

Figure 9 Ligand blot: Specific rMsEno binding to pECL proteins. Proteins of purified porcine erythrocyte lysate were separated by SDS-PAGE, subsequently blotted onto a nylon membrane and incubated with biotinylated rMsEno. Bound proteins were visualised using peroxidase-labelled streptavidin, and chemo luminescence. Four distinct pECL protein bands (signed by arrows on the film) were detected and identified as porcine leukocyte elastase inhibitor (~59 kDa), ß-actin (~45 kDa), GAPDH (~34 kDa), and S100 calgranulinB-like protein (~20 kDa). M, molecular weight marker (kDa).

8. Milestones and Outlook

M. suis causing infectious anaemia in pigs has been well known since the middle of the last century. Its unique lifestyle on or in porcine erythrocytes, its high metabolic adaptation to the extraordinary host cell and microenvironment and its uncultivability fascinate researchers.
Nevertheless, the application of classical microbiological tools is restricted since the propagation of these uncultivable bacteria in the host is often insufficient to gain detailed knowledge about their biology. The reclassification of *M. suis* to mycoplasmas in 2001 made new cultivation approaches according to culture systems of other cultivable mycoplasmas possible. The application of molecular biological methods presently available such as sequencing of shotgun libraries and the expression of recombinant proteins in heterologous systems enable clarification on the pathobiology of bacteria without *in vitro* cultivation systems.

Within the present Ph.D. thesis several empirical approaches were systematically applied in order to obtain the optimal culture medium, and to develop a culture system for the *in vitro* propagation of *M. suis.*

- Cultivation of *M. suis* in several *Mycoplasma* media resulted in a long-term maintenance at low stationary level.

- This study demonstrated, for the first time, cultured *M. suis* cells outside the porcine host. The application of suboptimal growth conditions for *M. suis* induces a transformation into nanoforms that are ultra microforms (nanotransformation). These nanoforms are organised in microcolonies.

- *In vitro* infection of porcine erythrocytes with *M. suis* cells, derived from culture, revealed that cultured *M. suis* remained infectious and, thus, viable.

At the beginning of this Ph.D. study, no complete genomic sequence data of *M. suis* were available. Therefore, the application of shotgun library sequencing, Southern blot hybridisation and *in silico* DNA analyses provided insight into metabolic capacities and transport systems of *M. suis*.

- Glucose is obviously one of the main energy sources of *M. suis*.

- *M. suis* competes for and scavenges glucose from the porcine blood due to the presence of a glucose-specific PTS^{glc} system and a complete set of glycolytic enzymes; this leads to life-threatening hypoglycaemia in affected pigs.

- Transport capacities of *M. suis* are rather restricted. The Sec pre-protein translocase and the phosphate-specific Pst ABC-transporter of *M. suis* represent two general transport systems that may be part of a complex network needed for virulence and for stress adaptation.

- The *M. suis* glycolytic enzyme α-enolase is a so-called moonlight protein with additional uncommon functions beyond its well-characterised metabolic function.

- *M. suis* α-enolase is a membrane-localised and surface-accessible protein with immunogenic and adhesive properties.

- Comparison of the data acquired in the present work to whole genome sequence data of *M. suis* [Oehlerking et al., 2011] and to recent work performed in our research group [Hoelzle, 2008] revealed the following *M. suis* pathobiology model:

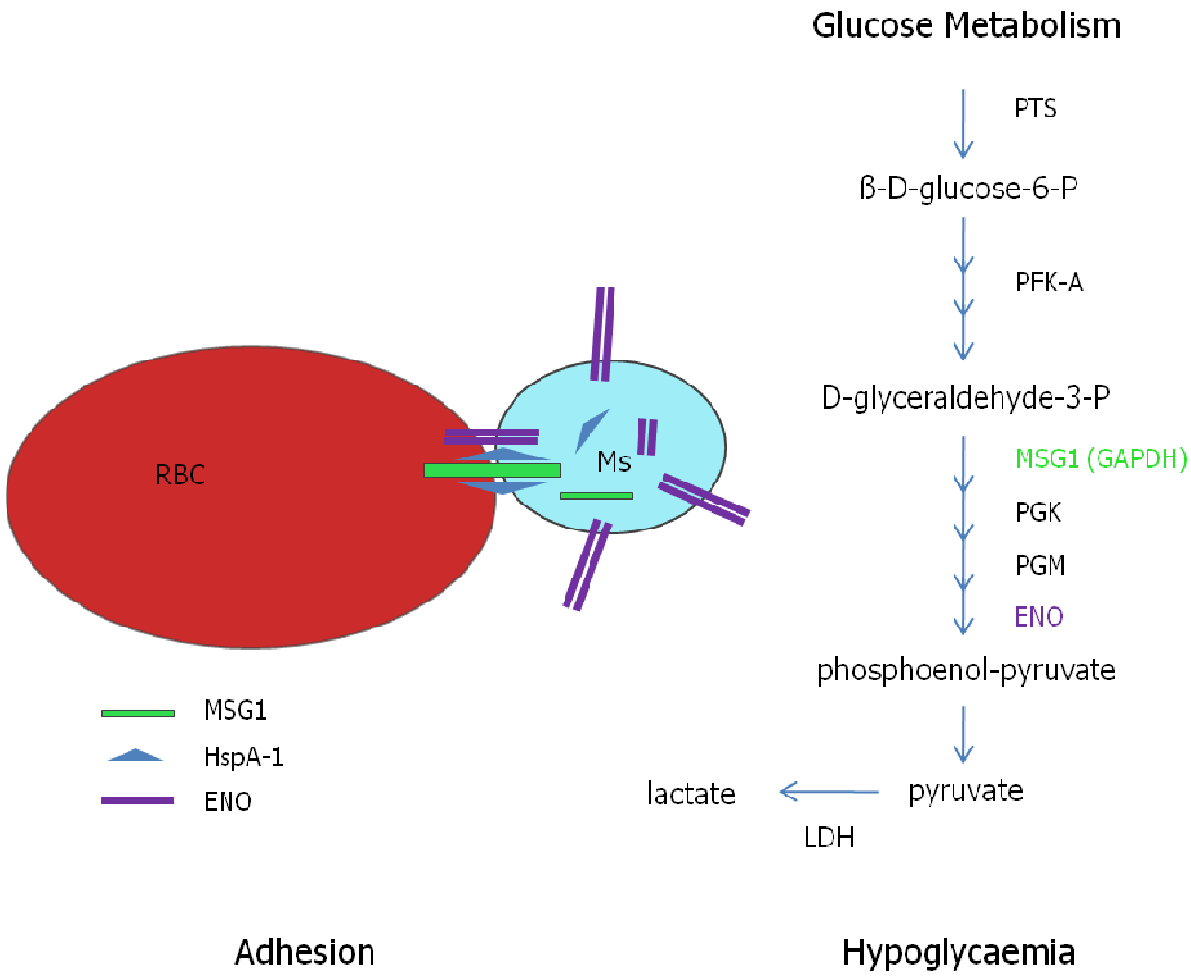

Figure 17. Model of glycolytic and multitasking *M. suis* proteins. A direct consequence of the glucose metabolism of *M. suis* is the severe hypoglycaemia in *M. suis*-infected pigs. Glycolysis-associated *M.suis* proteins that were found by shotgun sequencing are indicated. Finally, pyruvate is mainly converted into lactate by LDH (lactate dehydrogenase identified by genome annotation) leading to acidosis in the blood. In the present dissertation, *M. suis* α-enolase was characterised as surface-localised adhesin and is, therefore, considered to interplay with HspA-1 and MSG1 in a complex adhesion apparatus. Additionally, blood plasma components could be possible binding partners of *M. suis* α-enolase.

Future work will focus on the clarification of the metabolic adaptation of *M. suis* in combination with a detailed analysis of the whole genome sequences which were recently published.

The adhesion of *M. suis* is obviously crucial for its unique lifestyle on the erythrocytes. *M. suis* α-enolase is part of an adhesion complex; more detailed elucidation could be the basis for future vaccine development.

In the future, one vital ambition will be the replacement of animal experiments necessary for HM research. The present work provides first steps towards the establishment of an *in vitro* cultivation system for *M. suis* and other haemotrophic mycoplasmas.

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10. Curriculum *Vitae* and Presentations

Name	Sabrina A. Schreiner
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Nationality	Austria

Education

2007 - 2011	Post graduate studies and position as Ph.D. student at the Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Switzerland. Thesis «Analysis of *in vitro* Growth and Characterisation of the Metabolic Pathways of *Mycoplasma suis* Linked to Pathogenesis»
2006-2007	Diploma Thesis at the Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Switzerland. Thesis «Transport Mechanisms and Pathogenicity Factors of *Mycoplasma suis*: Analysis of Putative Transport Proteins»
2005	ERASMUS exchange studies in Microbiology (SS 2005), Swiss Federal Institute of Technology, Zurich (ETHZ), Switzerland.
2004 - 2007	Graduate studies in Molecular Microbiology and Biochemistry (Diploma), Leopold-Franzens University of Innsbruck, Austria.
2001 - 2004	Undergraduate studies in Biology, Leopold-Franzens University of Innsbruck, Austria.
1993 - 2001	General qualification for university entrance (Matura), Akademisches Gymnasium in Innsbruck, Austria.

Grants

2010	Travel grant by the Ph.D. Program in "Microbiology and Immunology" of the Life Science Zurich Graduate School - Doctoral School (LSZGS-GS), Zurich for attending the 18th congress of the International Organisation for Mycoplasmology (IOM) in Chianciano Terme, Italy.
2008	IOM travel grant for attending the 17th IOM congress in Tianjin, China.
2006 - 2008	Financial support by the 3R Research Foundation Switzerland. Project 104/06: "Development of *in vitro* strategies to propagate and characterise haemotrophic mycoplasmas"

Presentations of own research

2010

18th IOM Congress in Chianciano Terme, Italy. **Poster** entitled «α-Enolase - a Third Immunodominant Antigen in *Mycoplasma suis* - Infection?»

Biannual Meeting of the division of Bacteriology and Mycology of the German Veterinarian Association, Jena, Germany. **Poster** entitled «Kohlenhydrat-Stoffwechsel assoziierte Virulenzfaktoren bei *Mycoplasma suis*»

2009

7th Symposium of German-speaking mycoplasmologists, Morschach, Switzerland. **Poster** entitled «Etablierung eines *in vitro* Kultursystems für das hämotrophe Bakterium *Mycoplasma suis*»

Invited Talk in Salburger Group`s Seminar at the Zoological Institute (Evolutionary Biology) of University of Basel, Switzerland. **Talk** entitled «*In Vitro* Cultivation of *Mycoplasma suis*»

2008

15th Congress on Alternatives to Animal Testing, 12th Annual Meeting of Middle European Society for Alternative Methods to Animal Testing (MEGAT), University of Linz, Austria. **Poster** entitled «First *In Vitro* Cultivation System for *Mycoplasma suis*»

17th IOM Congress in Tianjin, China. **Poster** entitled «Development of an *In Vitro* Cultivation System for the Haemotrophic Bacterium *Mycoplasma suis*»

Biannual Meeting of the division of Bacteriology and Mycology of the German Veterinarian Association, Braunschweig, Germany. **Poster** entitled «Etablierung eines *in vitro* Kultursystems für das hämotrophe Bakterium *Mycoplasma suis*»

67th Annual Assembly of the Swiss Society for Microbiology, Interlaken, Switzerland. **Poster** entitled «Development of an *In Vitro* Cultivation System for the Haemotrophic Bacterium *Mycoplasma suis*»

2007

66th Annual Assembly of the Swiss Society for Microbiology, Interlaken, Switzerland. **Poster** entitled «Identification of Putative Transport Mechanisms in *Mycoplasma suis*»

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